



## Dynamics of chromosome segregation in *Escherichia coli*

Nielsen, Henrik Jørck

*Publication date:*  
2007

[Link back to DTU Orbit](#)

*Citation (APA):*  
Nielsen, H. J. (2007). *Dynamics of chromosome segregation in Escherichia coli*.

---

### General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

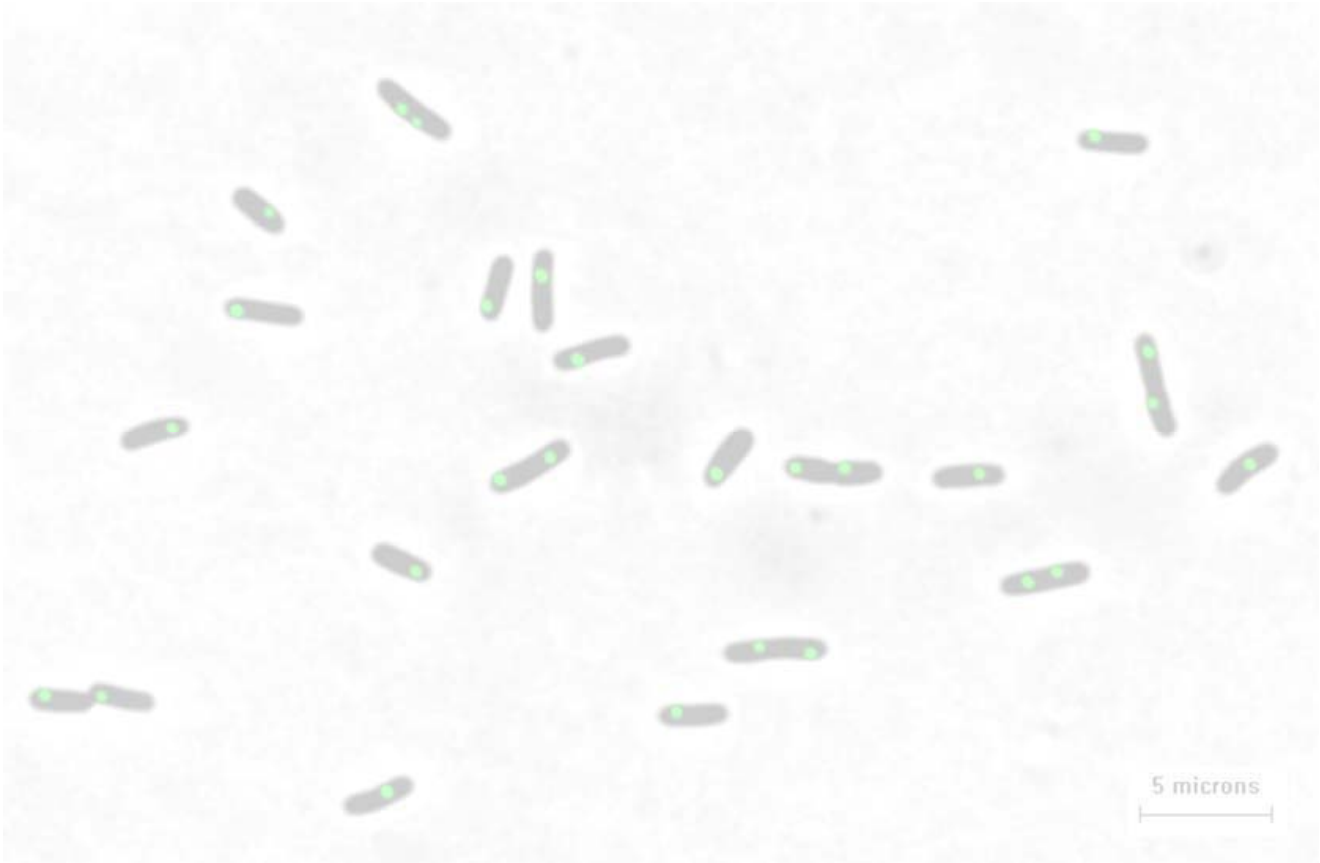
A grayscale fluorescence microscopy image of several *Escherichia coli* cells. The cells are rod-shaped and appear as dark, elongated structures against a lighter gray background. Inside each cell, two distinct fluorescent spots are visible, one colored cyan and the other yellow-green, representing the segregation of chromosomes. The cells are scattered across the frame, with some showing different orientations and positions of the fluorescent markers.

# Dynamics of chromosome segregation in *Escherichia coli*

Henrik Jørck Nielsen

BioCentrum - DTU  
2006

# Dynamics of Chromosome segregation in *Escherichia coli*



Ph.D.-thesis by  
Henrik Jørck Nielsen

October 2006  
BioCentrum  
Technical University of Denmark  
Kgs. Lyngby  
Denmark

*Picture on front page is from (Nielsen et al., 2006a) and was used as cover photo on Molecular Microbiology **61**(2) 2006.*

## **Preface**

This report is the result of my pre-doctoral work in the years 2003 to 2006 in Flemming Hansen's laboratory in Denmark and Stuart Austin's laboratory at the National Cancer Institute in the USA.

The study was sponsored by the Danish Government as a ph.d.-scholarship administered by the Technical University of Denmark. The study was supervised by Flemming G. Hansen, Biocentrum, DTU.

### **Acknowledgements**

I would like to thank my supervisor Flemming Hansen for excellent guidance and friendship during my 5 years in his laboratory.

I thank Dr. Stuart Austin (NCI) for his great abilities as mentor and excellent guidance during my many stays at NCI-Frederick. I am also grateful for the kindness and hospitality given to me by Stuart Austin and his lovely family at numerous occasions.

I thank the lab technicians here at DTU and at NCI Susanne Koefod and Brenda Youngren without which the results presented in this thesis could not have been produced.

Many thanks to my friend and former colleague Jesper Ottesen for help with proof reading of this thesis.

I thank the Danish Government and DTU for funding this study as well as the Otto Mønsted foundation, the Oticon foundation, the Poul V. Andersen foundation and the Frant Alling foundation for the additional fundings making my trip to NCI possible.

Finally I thank my wife and children for their support and understanding during many of the late hours.



## Contents

<b>Abstract.....</b>	<b>iii</b>
<b>Abstract in Danish / Resume på Dansk.....</b>	<b>v</b>
<b>List of publications.....</b>	<b>vii</b>
<b>Abbreviations and nomenclature.....</b>	<b>ix</b>
<b>1 Introduction .....</b>	<b>1</b>
1.1 The cell cycle	1
1.2 Applied chromosome labeling techniques	7
1.3 Models for Chromosome Segregation	9
1.4 Position of replication	14
1.5 Dynamics and organization of the replicating chromosome	19
<b>2 Results .....</b>	<b>25</b>
2.1 Counting and measuring cells	25
2.2 Optimizing the P1-par labeling system	26
2.3 Time-lapse studies and flowcells	28
2.4 Progressive chromosome segregation	30
2.5 Developing a pMT1- <i>par</i> labeling system	33
2.6 Separate replichores localize to separate cell halves	34
2.7 Stickiness	35
<b>3 Discussion .....</b>	<b>39</b>
3.1 A model for chromosome segregation	39
3.2 Perspectives	46
<b>4 Bibliography .....</b>	<b>511</b>
<b>5 Co-author statements.....</b>	<b>59</b>
<b>6 Papers and manuscripts.....</b>	<b>61</b>
Paper 1 An automated and highly efficient method for counting and measuring foci in rod shaped bacteria	6 pages
Paper II Progressive segregation of the <i>Escherichia coli</i> chromosome	11 pages
Supplementary material for Paper II	1 page
Paper III The <i>Escherichia coli</i> chromosome is organized with the left and right chromosome arms in separate cell halves	8 pages
Supplementary material for Paper III	3 pages





## **Abstract**

Since the 1960's the conformation and segregation of the chromosome in *Escherichia coli* has been a subject of interest for many scientists. However, after 40 years of research, we still know incredibly little about how the chromosome is organized inside the cell, how it manages to duplicate this incredibly big molecule and separate the two daughter chromosomes and how it makes sure that the daughter cells receives one copy each. The fully extended chromosome is two orders of magnitude larger than the cell in which it is contained. Hence the chromosome is heavily compacted in the cell, and it is obvious that structured cellular actions are required to unpack it, as required for its replication, and refold the two daughter chromosomes separately without getting them entangled in the process each generation.

The intention of the study was initially to find out how the chromosome is organized in the cell by labeling specific parts of it. Later the dynamics of chromosome segregation was included.

Investigating chromosome organization by labeling of specific loci was already a widely used technique when I started on this thesis, but the data acquisition and treatment was slow and generally poorly described. There was a great need for an automatic standardized method capable of identifying the number and position of fluorescent foci in cells on photomicrographs fast and precise. A major part of my three-year study was devoted to the development of such a procedure. The result which is described in the accompanying Paper I, is a macro (program) written for the image analysis software Image Pro Plus capable of measuring the physical outline of cells, counting the number of foci within, and measuring their intra-cellular position. 1000 cells are processed in 3 minutes.

The development of this fast and reliable method enabled us to start the analysis on the distribution of various chromosomal loci inside slowly growing cells. With the actual counting and measuring no longer being any problem we could easily analyze 14 loci distributed on the *E.coli* chromosome. More than 15.000 cells were analyzed in total. The results are described in the accompanying Paper II and show clearly that the chromosome is segregated progressively. An unexpected delay between the replication and segregation of markers was also observed and led to a new model on the timing of chromosomal segregation (the Sister Loci Cohesion Model). The results of Paper II also strongly

indicated that the chromosome is not replicated in a central factory but by separated and migrating replication forks. A result confirmed by others.

Finally we developed a new labeling system compatible with the existing labeling system based on the P1 *par* system. Using the new system, which is based on the pMT1 *par* system from *Yersenia pestis*, we labeled loci on opposite sides of the *E.coli* chromosome simultaneously and were able to show that the *E.coli* chromosome is organized with one chromosomal arm in each cell half. This astounding result is described in Paper III.

Adding the results of the thesis together with known data results in the following description of the chromosome dynamics of slowly growing *E.coli* cells:

The chromosome of slow growing cells is organized with the origin at the cell center when it is newborn. It has one chromosomal arm on one side of the center and the other chromosomal arm on the other side. The terminus is at the new pole but migrates to the center soon after cell division. Replication is initiated at the origin at the cell center. The duplicated origins stay together for a short while and then migrate to the cell quarters. As the origins migrate away from the center the replication forks split up too and are from this point found on opposite sides of the cell center but randomly distributed. Supposedly the forks track along the two chromosomal arms that are separated to each cell half. As the forks replicate the two arms, the duplicated loci stay together for a while at the non-central position where they were replicated. This delay is the same for all loci. Thus segregation is progressive at a rate comparable to the rate of replication but segregation is delayed with respect to replication. After the delay one of the replicated loci is segregated to the other side of the cell center and the other one stays where it is. This way of segregating the chromosome ultimately leads to the placement of the two arms of the chromosome on each side of the cell quarter. Finally the replication forks meet at the terminus in the cell center and the replication is complete. The terminus does not separate until cell division where after it migrates to the new cell center and the original configuration is re-established.

## **Abstract in Danish / Resume på Dansk**

Siden 60'erne har man forsøgt at klarlægge kromosomernes organisering og segregering i *Escherichia coli*. Men selv efter 40 år ved vi stadig meget lidt om hvordan kromosomet er organiseret inde i cellen, hvordan det lykkes cellen at duplikere dette meget store molekyle og separere de to datter-kromosomer, samt hvordan det sikres at hver dattercelle kun får ét kromosom hver. Det fuldt udstrakte cirkulære *E.coli* kromosom er over 100 gange større end cellen hvori det indeholdes. Kromosomet er derfor pakket godt sammen og det er indlysende at der må findes strukturerede cellulære processer der hver generation pakker kromosomet op når det skal repliceres og pakker de to datter-kromosomer sammen igen hver for sig uden at de bliver viklet ind i hinanden.

Formålet med dette projekt var oprindeligt at finde ud af hvordan kromosomet er organiseret inde i cellen, men blev senere udvidet til også at omfatte kromosom-segregationsdynamik.

Studier over kromosomets organisering i cellen ved hjælp af mærkning af specifikke kromosomale loci var allerede en udbredt metode da jeg startede mit Ph.D.-studie. Data opsamling og behandling var dog langsom og generelt dårligt beskrevet. Der var et udtalt behov for en automatisk og standardiseret metode til at identificere antal og lokalisering af foci i celler hurtigt og præcist. En stor del af mit 3-årige Ph.D.-studieforløb er gået med at udvikle en sådan metode. Resultatet som er beskrevet i Paper I er et program (makro) skrevet til det digitale billedebehandlings- og analyseprogram Image Pro Plus der er i stand til måle de fysiske dimensioner af celler og tælle antal af foci inden i samt måle disse foci's intracellulære positioner. 1000 celler bliver talt og målt på cirka 3 minutter.

Udviklingen af denne hurtige og pålidelige metode satte os i stand til at begynde analyser af positioneringen af forskellige kromosomale loci i langsomt voksende celler. Da det ikke længere var noget problem at tælle og måle et stort antal celler kunne vi nemt analysere 14 loci fordelt jævnt over *E.coli* kromosomet. Flere end 15.000 celler blev analyseret i alt. Resultatet er beskrevet i Paper II og viser med al tydelighed at kromosomet segregeres progressivt. En uventet forsinkelse i mellem replikation og segregation af kromosomet blev observeret og ledte til en ny model for timingen af segregationen af kromosomet (the Sister Loci Cohesion Model). Resultaterne præsenteret i Paper II

indikerer også at kromosomet ikke repliceres i en central fabrik (factory) men af separerede replikationsgafler i bevægelse. Et resultat bekræftet af andre.

Endelig har vi udviklet et nyt mærkningssystem til mærkning af kromosomale loci som er kompatibelt med det eksisterende system baseret på P1 *par* systemet. Dette nye system som er baseret på pMT1 *par* systemet fra *Yersenia pestis* har vi brugt til at mærke loci placeret på modsatte sider af *E.coli* kromosomet (hver side af origin) og vist at *E.coli* kromosomet er organiseret med en kromosomal arm i hver cellehalvdel. Dette utrolige resultat er beskrevet i Paper III.

Ved at sammenholde resultaterne fra dette Ph.D-studie med eksisterende data kommer jeg frem til følgende skitse for kromosomets organisering i langsomt voksende *E.coli*:

Kromosomet i langsomt voksende *E.coli* celler er organiseret med dets origin i midten af den nyfødte celle. Herfra går de to kromosomale arme (de to halvdele af det cirkulære kromosom) ud til hver sin side således at den ene arm er i den ene cellehalvdel og den anden arm i den anden cellehalvdel. Terminus er ved cellens nyeste pol men migrerer til midten af cellen kort tid efter celledelingen. Replikationen bliver initieret ved kromosomets origin i midten af cellen. De dupliserede origins forbliver sammen for en tid, hvorefter de migrerer i hver sin retning til de to kvartpositioner i cellen. Idet de to origins migrerer fra midten adskilles også de to replikationsgafler som indtil da har befundet sig i centrum af cellen. Herefter fordeles de temmeligt tilfældigt omkring midten men på hver side af den. De løber givetvis langs hver deres kromosomale arm i hver deres halvdel af cellen. Det dupliserede DNA som skabes efterhånden som de to replikationsgafler replicerer hver deres arm forbliver sammen for en tid der hvor de blev repliceret; ligesom det var tilfældet for de to origins. Denne forsinkelse i mellem replikation og separation er den samme for alle loci. Segregationen af kromosomerne er derfor progressiv med en hastighed der nøje svarer til hastigheden af replikationen skønt segregationen dog er forsinket i forhold til replikationen. Efter forsinkelsen vil ét af de replicerede loci segregeres til den anden side af cellecentrum hvorimod den anden bliver hvor den er. Det er således tilsyneladende kun den ene datter-streng der segregeres i hver cellehalvdel. Denne måde at segregere kromosomerne på fører til sidst til at de to kromosomarme i hver deres cellehalvdel placeres på hver deres side af cellens kvartposition. Til sidst mødes replikationsgaflerne ved terminus i midten af cellen og replikationen er tilendebragt. Terminus segregerer ikke før celledelingen, hvorefter den migrerer til midten af den nye celle og udgangspunktet for kromosomorganiseringen er genskabt.

## List of publications

The scientific results of this thesis have been described in the following papers referred to in chapter 1 mainly by author and year and in chapters 2 and 3 by their roman numerals.

- I. Nielsen, Henrik J. & Hansen, Flemming G. (2006). An automated and highly efficient method for counting and measuring foci in rod shaped bacteria. *Manuscript*.
- II. Nielsen, Henrik J., Li, Yongfang, Youngren, Brenda, Hansen, Flemming G. & Austin, Stuart (2006). Progressive segregation of the *Escherichia coli* chromosome. *Molecular Microbiology* **61**(2), 383-393.
- III. Nielsen, Henrik J., Ottesen, Jesper R., Youngren, Brenda, Austin, Stuart & Hansen, Flemming G. (2006). The *Escherichia coli* chromosome is organized with the left and right chromosome arms in separate cell halves. *Molecular Microbiology* **62**(2), 331-338.



## **Abbreviations and nomenclature**

GFP	Green Fluorescent Protein
CFP	Cyan Fluorescent Protein
yGFP	yellow Green Fluorescent Protein (see Paper III)

Early, intermediate, and late markers refer to markers that are replicated early, mid-way or late in the replication cycle. There is no specific boundary between them but in general the early markers refer to the first third of the chromosome that is replicated and the intermediate and later markers are the middle and last thirds respectively.





# 1 Introduction

In this chapter I present basic knowledge important for understanding chromosome dynamics. I begin with the cell cycle of bacteria. Then follows a description of the different methods used to label and follow chromosomal loci in the cell and a presentation of the current models on chromosome segregation. Looking at the position of the replication forks is of great interest when studying chromosome dynamics and is discussed separately in the following section. Lastly I describe the published data that have led to the different models on chromosome segregation and give a complete review of the present results and opinions on chromosome organization and segregation in *E.coli*.

I show how incompatible many of the reports on chromosome dynamics in *E.coli* are, and in a search for a consensus try to isolate the published data that in general is in agreement as well as sort out where the individual authors could possibly be wrong. From this and from our own results I conclude on the actual organization of the *E.coli* chromosome and establish new models that explains not only the latest results in the field but also many of the older results.

## 1.1 The cell cycle

The cell cycle refers to the cyclic progression of macromolecular events leading to cell division and to two basically identical daughter clones. These events repeat themselves in the daughter cells leading to division once again and four new clones one generation later. Therefore it is referred to as the cell cycle as it is a cycle of events that repeats itself in every cell from newborn to division. In a balanced culture where all rate coefficients are constant and equivalent, these events are basically the same in each and every cell, although the natural variation from clone to clone is significant (Koch, 1996). When discussing the cell cycle of *E.coli* often only the DNA replication is considered although the cell cycle embraces all events leading to the cell division. In this thesis too the emphasis will be on the parts of the cell cycle that involves replication as well as segregation of the DNA.

### 1.1.1 Defining the cell cycle

The cell cycle of the *E.coli* cell is essentially defined by the inter-initiation time (I), which is the time it takes for the cell to build up an initiation potential. This potential is reached when the cell reaches the initiation mass (Donachie, 1968); a mass that does not vary considerably with the growth rate (Koppes and Nanninga, 1980). Once the initiation mass is achieved the cell initiates DNA replication from all origins (Skarstad *et al.*, 1986), and the replication period (C) begins. This period is followed by the D-period which is the period from termination of replication to cell division. When the cell initiates replication it immediately begins building up the next initiation potential which will lead to the next initiation of replication after one inter-initiation time I. Hence in balanced growth the ‘*cell cycle is a cyclic achievement every I minutes of the capacity to initiate chromosome replication followed by cell division C+D minutes later*’ (Helmstetter, 1996), and the generation time  $\tau$  is dictated by and equal to the inter-initiation time I. Thus the events required for division often begins before the previous division (when  $C+D>I$ ).

At slow growth rates the cell cycle is very simple: At some time after cell division the initiation potential is achieved. When no DNA replication is ongoing in this period as it is the case at very low growth rates, this period is referred to as the B-period. Then the cell initiates its only origin, the B-period ends and the C-period begins. DNA replication is terminated C minutes later and the cell finally divides after further D minutes segregating two non-replicating chromosomes to each daughter cell. B-periods are often seen in *E.coli* B/r strains at slow growth but only observed for the K-12 strains at very slow growth (Michelsen *et al.*, 2003), for example in minimal succinate media.

When  $C+D$  equals I there is no B-period as cells initiate at cell division. If  $C+D>I$  and  $C<I$  initiation occurs before cell division but after termination of the previous round of replication. Hence replication is initiated from two origins and daughter cells receive a replicating chromosome but there is no overlapping replication, i.e. chromosomes do not have more than two replication forks at any time. This can be observed when *E.coli* K-12 grows in minimal glycerol media.

At moderate growth rates where  $C\geq I$  there will be ongoing replication in the cell at all times as the previous round of replication will not terminate before the next begins. Daughter cells will in this case receive well replicated chromosomes. Many *E.coli* strains show continuous replication ( $C\sim I$ ) when grown in minimal glucose media (Michelsen *et al.*, 2003). When  $C>I$  replication of chromosomes is initiated before the previous round is terminated resulting in multi fork replication, i.e. when the same chromosome is being replicated from several positions by 6 replication forks or more (as many as 14 forks can replicate from the same chromosome, at  $C\geq 2I$ )

At very high growth rates the replication of the chromosomes that are segregated to each daughter cell at cell division was initiated as much as 3 generations before. This example is true when realistically I, C, and D equals 20, 40, and 20 minutes. These cells are actually born with two separate chromosomes as the D-period leading to cell division starts at the previous cell division. Cells also initiate at cell division and are thus born with 8 origins but only 1-2 termini. Such an extreme situation can for some strains be obtained by growth in L broth supplemented with glucose.

The B-, C-, and D-periods of the bacterial cell cycle are often compared to the analogue phases of the eukaryotic cell cycle. One should be careful when doing such comparisons though. The D-period for example, which is the period from termination of replication to cell division, is often compared to the G2/M phase of eukaryotes, although they only share the fact that they lie in between termination of DNA replication and cell division. Bacteria don't show any resemblance with the eukaryotic chromosome partitioning process. Consequently one should be careful comparing the bacterial D-period with the eukaryotic G2/M phase. When  $C+D>I$ , initiation of the next C-period takes place during the D-period, which is not possible in eukaryotes. Hence, in this case, any resemblance to the eukaryotic G2/M phase is gone and comparison of the two becomes meaningless. For that reason, only the B, C, and D terms are used in this thesis when referring to phases of the bacterial cell cycle.

### **1.1.2 Initiation of replication**

Replication is initiated once and only once in the balanced bacterial cell cycle (Skarstad *et al.*, 1986). Initiation occurs when initiator proteins (DnaA) binds five 9-mer sequences known as DnaA boxes in the *OriC* region and create the initiation complex (Messer and Weigel, 1996). This happens when the cell reaches its initiation mass (Donachie, 1968). The initiation mass defined as the mass per origin where the cells initiate is constant for a given strain over a range of growth rates (Churchward *et al.*, 1981). Initiation occurs from all origins in the cell almost at once with an extraordinary precise timing (Boye and Lobner-Olesen, 1991). Furthermore every origin initiates only once. As important as it is that the cell initiates every cell cycle from all origins, just as important is it to ensure that newly replicated origins do not immediately re-initiate but wait until the next cell cycle. This is regulated by the Dam and SeqA proteins.

The function of the SeqA protein is to bind newly formed origins after initiation of replication and protect them from further initiation; a process called sequestration (Slater *et al.*, 1995). This process is part of the initiation mechanism that ensures that every origin is initiated once and only once when the initiation mass of the cell is achieved. SeqA

recognizes and sequesters the origins because the newly formed daughter origins, as well as newly formed DNA in general, are hemi-methylated at GATC sites. GATC sites are found throughout the chromosome of *E.coli* and normally methylated at the N<sup>6</sup> position of the adenines on both strands by the methyl transferase enzyme Dam (Bakker and Smith, 1989). Newly synthesized DNA formed during replication is only methylated on one strand because the other has just been created and not yet methylated. SeqA recognizes these hemi-methylated GATC sites and binds to them (Fujikawa *et al.*, 2004), preventing a second initiation event at the origin (von Freiesleben *et al.*, 1994). Eventually Dam will re-methylate these GATC sites, but at that point the initiation potential has dropped because DnaA (the initiator protein) has been titrated by high affinity DnaA boxes on the newly formed chromosomes (Hansen *et al.*, 1991). The time window where origins are sequestered and protected from re-initiation is referred to as the eclipse period and defines the theoretical minimal length of the inter-initiation time I. As expected the eclipse period shortens if Dam methylase is over expressed, indicating that the eclipse corresponds to the period of origin hemi-methylation (von Freiesleben *et al.*, 2000).

SeqA has two functional domains. An N-terminal multimerization domain (residues 1-50) and the C-terminal DNA binding domain (residues 51-181) (Guarne *et al.*, 2002). It binds DNA as a dimer and oligomerizes on the DNA. Both features, the DNA binding as well as the ability to oligomerize, is important for the proteins function in initiation regulation *in vivo* (Guarne *et al.*, 2005). As expected a strain deleted for either the SeqA or Dam proteins is asynchronous in its initiation of DNA replication as it is impaired in its ability to prevent re-initiation of newly formed origins (Boye *et al.*, 1996; Boye and Lobner-Olesen, 1990).

### 1.1.3 Elongation

Once the replication has been initiated two so-called replication forks are formed at each origin. The replication forks replicate one arm of the chromosome each going bi-directionally from the origin and meeting in the terminus region. The term ‘fork’ is used because one double strand of DNA is coming in and two are coming out of the replication complex, thus forming a fork of DNA. The replication speed is constant from initiation to termination under normal conditions (Atlung and Hansen, 1993).

The main component of the forks besides of course the DNA is the polymerase III holoenzyme which does the actual strand synthesis. There are two active holoenzymes, each synthesizing one new daughter strand using one of the parental strands as template (semi-conservative replication). In front of the holoenzymes the DNA is melted by DnaB and single stranded DNA is protected by the single strand DNA binding protein SSB until

it reaches the polymerase. As the polymerase III can only add deoxy ribonucleotides to the 3'-hydroxyl end of the DNA, there will be a leading and a lagging strand. The leading strand is the 5'-3' strand that is continuously replicated. The other strand is in the 3'-5' direction and is replicated discontinuously in so-called Okazaki fragments before they are ligated (Okazaki and Okazaki, 1969). Before and after the forks topoisomerases act to release the helical tension created by the replication.

Knowing the physical position of the replication forks in the cell is important for clarifying the spatial dynamics of chromosome replication and segregation and is discussed separately later.

#### **1.1.4 Termination of replication**

Termination occurs when the replication forks collide in the TerC region of the chromosome opposite to the OriC. *ter* sites in the terminus region ensures that one fork do not go through the terminus region but stop and wait for the other fork (Pelletier *et al.*, 1988). Upon termination the two completed chromosomes will be interlinked, or catenated (Sundin and Varshavsky, 1981). Before they can be segregated they have to be decatenated. This is done by topoisomerase IV (Deibler *et al.*, 2001). Occasionally sister chromosomes will recombine and form one dimeric structure. This has to be resolved into two separate chromosomes before the chromosomes can segregate. Resolution happens at the 28 base pair recombination site *dif* site in the terminus region by the XerC and XerD resolvases (Sherratt *et al.*, 2004). FtsK is responsible for recruiting the resolvases to the *dif* site (Massey *et al.*, 2004). FtsK is a very large 1329 aa protein that is vital for the cell. It consists of two domains separated by a long ~700aa linker. The ~500 aa C-terminal domain activates the Xer recombination complex in a ATP-dependent manner. It is however the ~200aa N-terminal membrane spanning domain of the protein with unknown function that is vital for the cell (Wang and Lutkenhaus, 1998).

#### **1.1.5 Determining cell cycle parameters**

The cell cycle was originally measured using synchronized cells (Helmstetter and Cummings, 1963). Synchronized cells are all at the same point in the cell division cycle; all initiating at the same time and all dividing at the same time etc. Hence by taking samples from a culture of synchronized cells at different points in time the variation in cell size and DNA content through the cell cycle can be determined.

Synchronized cells can be obtained from so-called baby machines. A baby machine is as it implies a machine that produces newborn 'baby' cells. A popular technique used in baby-machines for much of the work on bacterial cell cycle research is the membrane

elution technique. Cells are attached to a nitro cellulose membrane, optionally coated with poly-D-Lysine. The immobilized cells grow and divide normally on the filter when continuously flushed with fresh media releasing newborn cells into the effluent (Cooper and Helmstetter, 1968; Helmstetter *et al.*, 1992; Helmstetter and Cummings, 1963). These newborn cells are then collected and grown in small batches. Cell growth and division are measured with standard techniques (optical density, colony forming units etc.) and DNA synthesis periods and synthesis rates are determined by for example measuring the incorporation of radioactive or fluorescent nucleotides. These kind of experiments were popular in the 70'es and revealed most of the basic knowledge on the bacterial cell cycle (Helmstetter and Pierucci, 1976; Pierucci and Helmstetter, 1976)

Today bacterial cell cycle parameters are nearly always measured using a flow cytometer. In the 80'es flow cytometry became sensitive enough to be used on bacteria. In the flow cytometer cells are flushed in a water beam rapidly across a microscopy slide. Before the cells are put in the flow cytometer they are fixed and the DNA is labeled with fluorescent dyes. As they pass across the slide in the flow cytometer they are exposed to a beam of exciting light and fluorescence is measured for each cell as well as light scatter. These two values are directly proportional to the DNA content and the cell size respectively. Methods were developed to use this technique on *E.coli* revealing detailed information on the relationship between DNA content and cell size (Boye *et al.*, 1983). Using computer simulations of the cell cycle based on the knowledge of the cell cycle obtained from the early experiments using synchronized cultures and fitting these to experimentally obtained DNA distributions from a flow cytometer it ultimately became possible to analyze a sample of exponentially growing cells and determine the length of the C, D and B periods directly (Skarstad *et al.*, 1985).

The implementation of flow cytometry made it possible to take a sample from any exponentially growing culture in any experiment and determine the cell cycle parameters for that particular culture. That has been used in this work to verify that any culture used for a chromosome segregation study is growing normally and to determine the C and D periods directly in that culture, and not from some other experiment.

The model used in this work to simulate the cell cycle parameters from the DNA distribution of exponentially growing cells and to determine the length of C and D periods is slightly different from the one used and described by Skarstad (Skarstad *et al.*, 1985). Skarstad assumed that the coefficient of variation is the same for all measured DNA contents. It has however been shown recently that this assumption is possibly incorrect (Michelsen *et al.*, 2003); instead there is a described linear correlation. Changing the assumption on the variation of DNA content gives better simulations and better

determinations of the cell cycle parameters (Michelsen *et al.*, 2003). This modified version of Skarstad's original model has been used in the present work.

## **1.2 Applied chromosome labeling techniques**

Most of the present knowledge on bacterial chromosome dynamics is based on the development 10 years ago of techniques for labeling chromosomal loci inside the cell, techniques that practically revolutionized the field. They have been used to visualize the position of the origin, the terminus or other markers in cells under different conditions. The results from these labeling experiments form the basis for all of the models on chromosome segregation. Here follows a short description of them including the one used in this work.

### **1.2.1 The repressor / operator system**

In 1997 the first system capable of visualizing the position of specific parts of the chromosome inside the living cell was published for *Bacillus subtilis* (Webb *et al.*, 1997) and later the same year for *E. coli* (Gordon *et al.*, 1997). The authors inserted 256 tandem repeats (Straight *et al.*, 1996) of the lactose operon operator into the chromosome near the origin or terminus of replication. Then they fused the green fluorescent protein GFP to the *lac* repressor LacI and expressed the fusion protein from a plasmid. The repressor fusion protein bound the operator repeats and resulted in green fluorescent foci at either the origin or the terminus. This was the first time specific and discrete DNA loci had been visualized in living cells of *E. coli*. The system was however quite genetically unstable as the tandem repeats tended to cross out by homologous recombination. For that reason the first system was developed in a strain incapable of doing any homologous recombination. A system was later developed without this problem by inserting 10 bp of random sequence between 240 repeats (Lau *et al.*, 2003).

This system is only capable of visualizing one locus, or alternatively more loci but all using the same color. The single color restriction of the Lac operator/LacI-GFP system was later circumvented by using it in combination with a similar system using the tetracycline operator and repressor (Lau *et al.*, 2003) or a system using the phage lambda cI repressor/operator (Fekete and Chattoraj, 2005).

### **1.2.2 Fluorescent In Situ Hybridization**

In 1998 the first results using Fluorescent In Situ Hybridization (FISH) to visualize the DNA loci was published from Hiraga's lab (Niki and Hiraga, 1998). The method uses

specific fluorescent DNA probes that are hybridized to the chromosomal DNA inside fixed and gently lysed cells. This method has the advantage that several distinct loci can be labeled at once. On the other hand a major downside is that cells have to be fixed and are completely dead introducing the possibility of artifacts and excluding the possibility of doing time-lapse experiments. The FISH technique was soon adapted to other organisms such as *Caulobacter crescentus* (Jensen and Shapiro, 1999) and used with success. The *lac* operator/LacI-GFP system was eventually made for the *C. crescentus* too and actually showed to produce results comparable with the FISH method (Viollier *et al.*, 2004).

### 1.2.3 P1 partitioning system

A third system was developed and published in 2002 (Li *et al.*, 2002). This system is based on the partitioning system of the plasmid phage P1. The plasmid contains a sequence *parS* that is bound by the P1 encoded ParB protein. The ParB protein spreads out from the *parS* to the adjacent DNA. Hence when labeled with GFP, ParB forms fluorescent foci inside the cell if a *parS* sequence is present. ParA binds to the ParB and is required for normal partitioning, but Li *et al.* made a truncated version of the ParB removing the first 30 amino acids from the protein making it incapable of binding to the ParA protein, though still capable of binding *parS* and forming foci in the cell.

This method has the advantage over the FISH method that it works in living cells. It also has an advantage over the repressor/operator system since it is completely genetically stable, and the *parS* site is small and easily inserted in any strain (286 bp for the *parS* containing insert compared to 7440 bp for 240 *lacO* repeats). On the downside however it only allows one color, so only one locus can be visualized at a time.

Very recently a similar system using the pMT1 ParB and *parS* was developed in our lab (Nielsen *et al.*, 2006). This system is completely compatible with the P1 labeling system and thus provides the possibility of visualizing two different loci with two different colors at the same time.

### 1.2.4 Data acquisition

When labeling specific loci of the chromosome using any of the techniques described above the cells have to be analyzed by fluorescence microscopy in order to produce images that can be measured for intracellular positions of the labeled DNA. Usually a combination of phase contrast and fluorescence microscopy is used. The first shows the outline of the cell very clearly, and when the fluorescent image is overlaid on the phase contrast image the exact position of the foci inside the cell can be easily measured.



Phase contrast microscopy is very suitable for this purpose as it produces clearly defined cells with high contrast and does not have the problem of creating shadows as Differential Interference Contrast microscopy does. Alternatively membrane dyes can be used and thus foci as well as the cell outline determined by fluorescence microscopy alone. This is theoretically a better solution as it reveals the true outline of the cell. In phase contrast microscopy the cell to background boundary, which is the one used for measurements, is not necessarily the same as the true cell outline because of the ‘Halo’ effect of phase contrast microscopy. This is nevertheless the preferred method. Cell membrane dyes can arguably interfere with cell physiology although that would not be a problem in FISH experiments.

The inaccuracy in using phase contrast microscopy for determining cell outlines can be minimized and at least kept constant for all cells by maintaining a high level of cell to background contrast and by defining the boundary between cell and background using a threshold value calculated on the basis of this contrast value consistently for all cells (Paper I). Unfortunately it is usually not reported how the cell outline is determined in experiments using phase contrast microscopy. Hence it is difficult to know how much variation in the end result is introduced from this step.

Measurements of the position of foci inside the cell consist very often only on measuring the distance from one pole to the center of each focus. These measurements are almost always done manually, aided by some software (MetaMorph, Object Image, IP Lab Spectrum, Image Pro Plus etc.). The user will manually determine where the poles and foci centers are, and the software will then calculate the pole to foci distances. The method of determining where the poles and foci are, if any, is never reported. What is the pole? Is it the very end of the cell, the point where it starts converging, or somewhere in between? That is usually decided by the person operating the software and therefore a lot of variation is expected to be introduced in this step.

As a part of this thesis I have developed a fully automatic method of measuring cell outline, size, and position of foci. Not only does that minimize the variations associated with the manual methods mentioned above, but more importantly it speeds up the process tremendously. 1000 cells are measured in less than five minutes, a task that would take at least 4 hours using the old manual method. The method is described in Paper I.

### **1.3 Models for Chromosome Segregation**

In the following the different major models presented by scientist in the field of bacterial chromosome segregation during the last 10 years is presented. Only a brief and general

description is given as details on the results supporting or disputing the different models are reviewed in later chapters.

### 1.3.1 The Extrusion-Capture Model

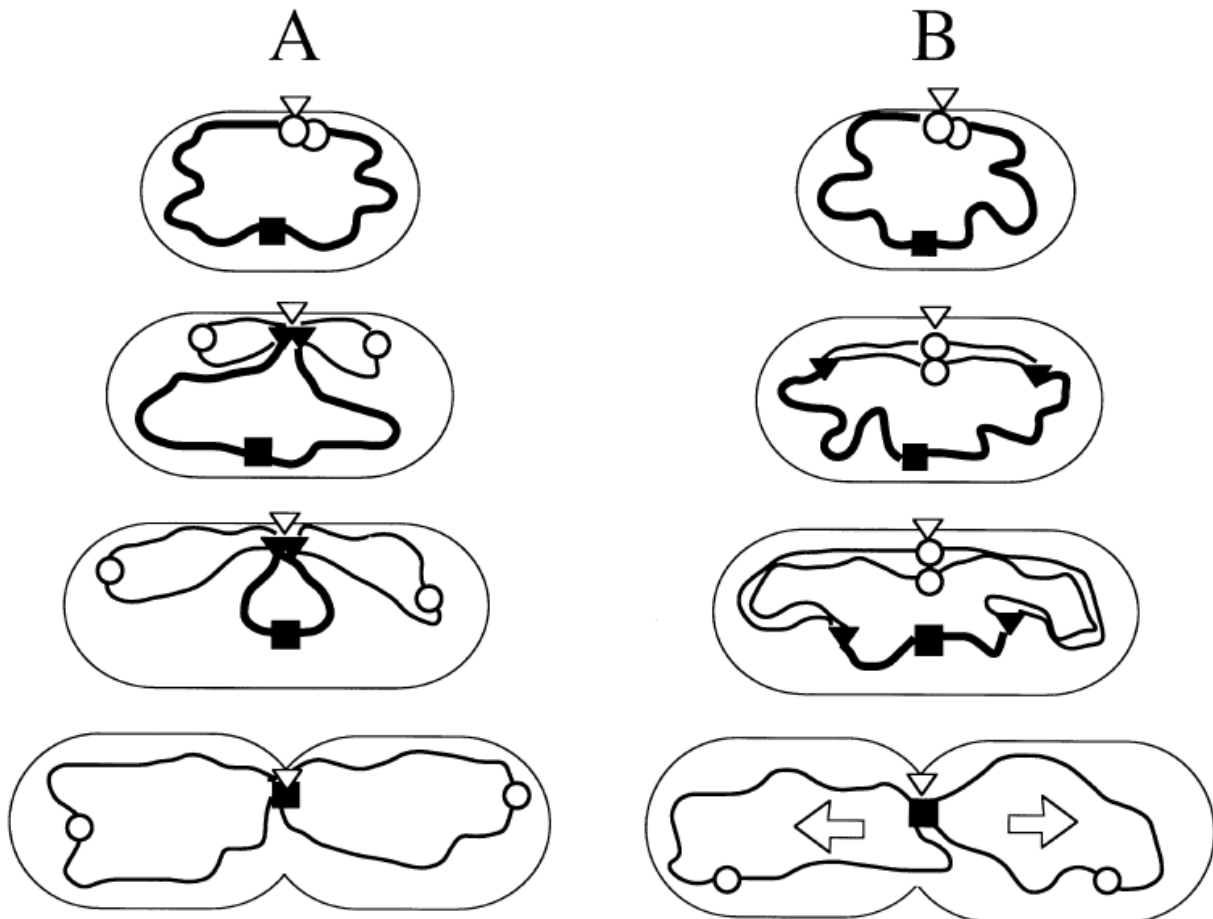
This model was originally described in 1974 (Dingman, 1974) but was refined and given the present name in 2001 (Lemon and Grossman, 2001). Basically it assumes that much of the force necessary for separating the two daughter chromosomes is provided by replication itself. The nascent chromosomes forming in the trail of the replication are completely relaxed, unfolded, and untangled. This is therefore the perfect time to separate the chromosomes. For many years the DNA polymerases was thought to track along the DNA inside the cell, replicating the DNA along the way. This model however assumes that the two replication forks involved in the bi-directional replication of the chromosome are linked together and positioned in the center of the cell (Figure 1.1A). The forks stay there throughout the replication pulling the DNA in for replication and pushing, or Extruding, the nascent chromosomes out. This is the central replication factory. If the replication factory is in fact held in place by somehow anchoring it to the cell membrane or some structure present at the cell center, the assumption is liable that it could progressively pull the entire chromosome through the factory (Lemon and Grossman, 2001). In order for segregation to take place it is important that the forming chromosomes are directed to opposite cell halves. The Capture-Extrusion model state that if only the origins are directed away from the middle and Captured at the quarter positions the rest of the DNA will automatically follow, condensing around the captured origins and eventually form the new nucleoids. Hence the most important events in this model is the replication at a central replication factory, the directed extrusion of the newly formed DNA away from the factory and the capture and holding of the chromosomes (see (Sawitzke and Austin, 2001) for a review).

### 1.3.2 The Sister Chromosome Cohesion Model

In opposition to the widely accepted Extrusion-Capture model Hiraga and coworkers have proposed the Sister Chromosome Cohesion Model (Hiraga *et al.*, 2000; Sunako *et al.*, 2001). This Model describes the segregation process in a way that is much more similar to the segregation of eukaryotic chromosomes. Chromosomes are thought to stay paired together for the entire or much of the replication period and then actively segregate as a unit before the cell divides (Figure 1.1B). This would require an additional and so far unknown segregation mechanism in the cell. Mitotic like spindles have been proposed but no evidence for their existence presented. The consensus seem to be that chromosomes

stay cohered together 1/3-2/3 of the C-period (Molina and Skarstad, 2004; Sunako *et al.*, 2001). They are then separated before the rest of the replication runs to termination.

An implication of this model is that the replication forks do not have to be centrally located; in fact they would be expected not to be but to track along the DNA. Determining the location of the replication forks has therefore been an important factor in finding the correct model and will be discussed separately.



**Figure 1.1 Two models for chromosome segregation.**

A. The extrusion–capture model: after initiation from the central ‘factory site’ (open triangle) the origins (circles) move out toward the poles followed by the newly replicated sequences (thin lines). Unreplicated DNA (thick line) is fed into the factory, and the terminus (square) is drawn to the cell centre toward the replication forks (closed triangles). Chromosome markers are segregated progressively as they are replicated, finishing with the terminus.

B. The sister chromosome cohesion model: after initiation, the sister sequences cohere and become paired along their length as they are replicated. Late in the cell cycle, the origin and other markers segregate together. One version of the model is drawn. In a variant, the sister regions pair as shown, but the replication forks remain at the cell centre (Hiraga *et al.*, 2000).

Figure and text taken directly from (Li *et al.*, 2002)

### 1.3.3 The Sister Loci Cohesion Model

This is the model proposed by the author of this thesis based on the results of this work and presented in the accompanying Paper II. It is to some extent a hybrid of the two previous

models. It only considers the temporal relationship between replication and segregation. The spatial relationship is described next in the Home and Away Segregation Model.

Duplicated loci stay paired together for some time after replication. After a delay they are then segregated to opposite sides of the cell center. This delay is constant for all loci, so that segregation is progressive and happens at the same rate as the replication but with a temporal offset equal to the delay. During this delay the cell has time to do any repair and other recombinational activities that require two homologous double stranded DNA molecules. Once separated the two chromosomes do not go back across the middle.

This model resembles the Sister Chromosome Cohesion model in that they both suggest cohesion of daughter chromosomes. However where the later propose that the cohesion is maintained for the entire length of the chromosomes and then lost at once as chromosomes are separated as units this model suggests that sister cohesion is lost progressively from the origin towards the terminus following replication but delayed with respect to it.

The idea of progressive segregation on the other hand resembles the Capture-Extrusion model. There is a major difference though. As the segregation of loci are delayed significantly compared to their replication in this model the process of replication is not likely to drive the segregation. Hence another so far unknown segregation mechanism is needed.

#### **1.3.4 The Home and Away Segregation Model**

This model is also a result of the present work. It is based on the results presented in the accompanying papers II and III as well as the recent work of Wang and coworkers (Wang *et al.*, 2005).

The chromosome is organized with the origin and the terminus at the middle, one arm of the chromosome in one half of the cell and the other arm in the other half (Nielsen *et al.*, 2006; Wang *et al.*, 2006). Since the origin is in the middle initiation occurs here. As replication progresses the forks separate and migrates in opposite directions following the organization of the chromosome and ends up in separate cell halves. They eventually come back to the cell center at termination of replication as the terminus is located here. The replication forks track along the DNA duplicating loci at the intracellular position in which they are located. As described previously for the Sister Loci Cohesion Model, loci are thought to stay together for a while before they segregate although that is not critical for this model. Once sister loci segregate one stays where it is, this is the Home locus, and the other is taken to the other half of the cell - that is the Away locus. The Away locus is put just on the other side of the cell center on the inside of the DNA already present in that cell

half. The segregation pattern is the same for the other arm of the chromosome replicated by the other fork in the opposite half of the cell. The pre-division cell will thus have two chromosomes, one in each cell half. They each consist of two arms: One that stayed Home and one that came from the fork in the other half of the cell (the Away-copy of the other chromosomal arm). The Home loci are close to the old poles and the Away loci are close to the division septum. Thus the original configuration is restored.

## 1.4 Position of replication

The identification of the position of the replication forks is important for testing the models described above. If the forks are not located together in a central factory throughout the replication period, basic central factory models as the Extrusion-Capture model are incorrect. Note that the position of the replication forks has not been under investigation directly in this thesis, but speculations about their positions can be inferred from the results.

### 1.4.1 Visualizing the replication forks directly

Lemon and Grossman have visualized the PolC subunit of the replisome in *Bacillus subtilis* presenting evidence supporting the central factory model. The position of the replication apparatus was visualized directly using a fusion protein consisting of the catalytic subunit PolC fused to the fluorescent protein GFP (Lemon and Grossman, 1998). This PolC-GFP protein supported DNA replication *in vivo* and localized as discrete foci in the cell only when the cell was replicating its DNA. In slowly growing cells there was mostly only one focus that always localized to the middle of the cell. Some cells had 2 foci which localized to the quarters. At faster growth rates cells had more foci, but they always localized as one focus in the middle, two foci at the quarters or a combination with one focus at each quarter and one in the middle. These data suggested that replication takes place in a stationary replisome in the middle of the cell and that the DNA is pulled through, as originally proposed by Dingman (Dingman, 1974). Lemon and Grossman have further presented proof of this theory by looking at the position of a specific chromosomal region where the replication was blocked (Lemon and Grossman, 2000). The DNA in this replication block localized to mid-cell and was shown to co-localize with the DNA polymerase *tau* subunit. Upon release of the replication block the chromosomal region duplicated and migrated to the cell quarters.

However, as correctly pointed out by Hiraga and coworkers (Hiraga *et al.*, 2000), the results by Lemon and Grossman can easily be re-interpreted as replication forks separating from the middle to the quarters. Since Lemon and Grossman do not relate their results to the cell cycle and cell length or in other ways satisfyingly justify that the two PolC-GFP foci could not have represented only one replicating chromosome, their results can be interpreted as either fixed or separating and migrating replication forks. Similarly the replication block results can be challenged and claimed to support the sister chromosome cohesion model. Since the locus under investigation is rather close to the origin it is likely to replicate at the middle of the cell according to models with migrating replication forks

as well. Also, we question the conclusion that replication takes place in the middle just because the blocked locus and its associated replication fork are found in the middle. It is possible that segregation takes place in the middle and that the replication block causes the stalled fork to get stuck in the segregation apparatus located in the middle of the cell. A hypothesis supported by results from our lab on studies of segregation blocks (see section 2.7).

These challenges to the results of Lemon and Grossman emphasize the importance of knowing the exact cell cycle in the cells under analysis. The position of forks has to be related to the progress of the replication in order to convincingly claim that forks are either fixed or migrating. An observation of two foci when  $C+D < I$  is clear evidence for migrating forks whereas if  $C+D > I$  it depends on the cell length of the cells containing two foci as pointed out by Hiraga and coworkers. As it will be described in later sections, results concerning the dynamics of the chromosome are disturbingly often published without determining the basic cell cycle parameters.

Bates and Kleckner visualized the polymerase directly in synchronized *E.coli* cells (Bates and Kleckner, 2005) with a DnaX-GFP fusion protein developed by Andrew Wright (Tufts University, Boston). In this study the cell cycle was determined and the authors showed that virtually all forks (DnaX-GFP foci) came a part 1/3 into the C-period. The separated forks localized rather haphazardly between the cell quarters. The cells were grown in minimal succinate media with  $C+D < I$ . Thus the results can only be interpreted as replication forks migrating away from each others.

#### **1.4.2 Using SeqA as marker for the replication forks**

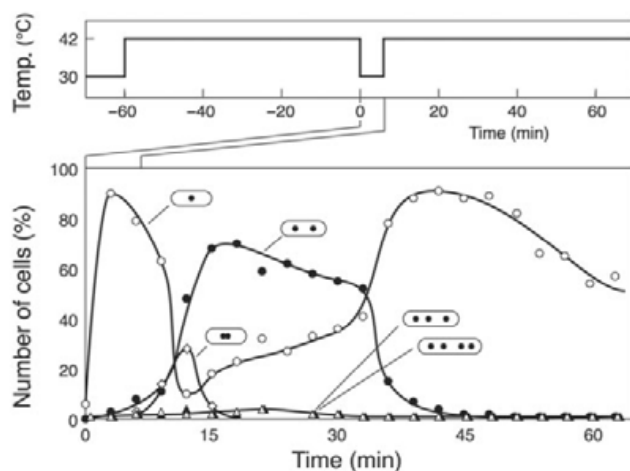
SeqA does not only bind GATC sites in the origin as described earlier. Other hemi-methylated GATC sites spread evenly throughout the chromosome are bound when properly spaced. SeqA binds a hemi-methylated GATC sequence if another sequence like it is present close to it on the same piece of DNA within three helical turns (Brendler and Austin, 1999). Furthermore binding only occurs when the two sequences follow specific spacing rules where optimal binding occur at spacings of 7, 12, 21, and 31 bp (Brendler *et al.*, 2000). Once bound further SeqA can oligomerize on to the SeqA-DNA complex.

During the replication of the chromosome tracts of hemi-methylated DNA strands are formed behind the replication forks. The length of these tracts depends on the rate of re-methylation by the Dam protein. Using kinetic data from the work of Campbell and Kleckner, Brendler *et al.* calculated that these tracts should consist of ~100 suitably spaced pairs of GATC sites (Brendler *et al.*, 2000). Using immunostaining of fixed cells (Molina and Skarstad, 2004; Yamazoe *et al.*, 2005) or a GFP-SeqA fusion protein in living cells

(Brendler *et al.*, 2000; Onogi *et al.*, 1999) these tracts can be visualized as foci in the cell. SeqA only forms foci in actively replicating cells (Hiraga *et al.*, 2000). As expected the presence of Dam methylase is also required for formation of foci (Onogi *et al.*, 1999) The number of foci depends on the growth rate and can be anywhere from 0 (no replication) to 16 (Brendler *et al.*, 2000; Molina and Skarstad, 2004). Each focus probably represents one of these tracts.

As these tracts consist of newly formed DNA and newly formed DNA is expected to be found mainly at the replication forks, the SeqA foci are generally thought co localize with the replication forks. This assumption seems to be true as the SeqA foci co localize with newly formed DNA as shown by pulse labeling the DNA with 5-bromodeoxyuridine (BrdU) (Adachi *et al.*, 2005; Molina and Skarstad, 2004).

The reports on numbers and distribution of these clusters of SeqA molecules in the cells vary considerably. At a moderate growth rate in glucose minimal media scientists agree that cells have no foci or one SeqA-focus when newborn. This focus then seems to split up in two foci that jumps rapidly to the cell quarter positions (Brendler *et al.*, 2000; Hiraga *et al.*, 1998; Hiraga *et al.*, 2000) as confirmed by time-lapse microscopy (Onogi *et al.*, 1999). At faster growth rates in rich media the number of reported foci depends somewhat on the technique (GFP fusion or immunostaining) as well as the reporting laboratory. Accordingly Onogi *et al.* observed 2 to 4 SeqA-foci in cells growing in glucose media supplemented with amino acids (Onogi *et al.*, 1999) where Molina and Skarstad have reported up to 8 in a similar media (Molina and Skarstad, 2004). Brendler and Austin reported as many as 14 SeqA-foci in some cells growing in L media with the majority of cells having 4 to 8 foci (Brendler *et al.*, 2000). Onogi *et al.* saw only 2 to 4 foci in a time-



**Figure 1.2 Separation of replication forks.**

Upper figure represents three-step temperature shifts for synchronous initiation of only one round of chromosome replication. Lower figure represents the number of cells with SeqA foci. Approximately 300 cells were counted in each sample. Open circles, cells with one SeqA focus; open diamonds, cells with two closely located SeqA foci; solid circles, cells with two separated SeqA foci; open triangles, cells with three SeqA foci; solid triangles, cells with four SeqA foci.

Figure and text taken from Yamazoe *et al.*, 2005.

lapse study of cells growing in similar media (Onogi *et al.*, 1999).



It is difficult to conclude anything about the number and positions of separated replication forks when reports vary so much, and accordingly most of these experiments really have not lead to any insight on whether forks come apart or not.

One convincing study used synchronized cells (Yamazoe *et al.*, 2005). The strain used had a temperature sensitive *dnaC* mutation allowing it to only initiate DNA replication at permissive temperature. By keeping the culture at non-permissive temperature and shifting it to permissive temperature briefly every 60 minutes, the cells become very synchronous in their replication. After 3 cycles most cells have only one chromosome which initiates at the shift to permissive temperature leading to cell division one generation later. After one such initiation, the number and position of SeqA foci were followed over time for one generation by immunostaining (Figure 1.2). At initiation there was one central focus, this then split up after 12 minutes (at 42 degrees) into two foci that migrated rapidly to the quarters. After 40 minutes the replication was terminated and the two foci merged in the middle as one. As there where only one round of replication initiated from a single origin each focus must have represented one replication fork and the results thus support a separating replication forks model.

### 1.4.3 Visualizing newly replicated DNA in the cell

Newly synthesized DNA in the cell has been visualized by pulse labeling the chromosome with 5-bromodeoxyuridine (BrdU). Subsequent fixation of the cells and immunostaining of the BrdU containing DNA reveals the position of the newly replicated DNA. In one study using this method newly replicated DNA was found either in the middle or at the quarters of the cell (Adachi *et al.*, 2005) very similar to many of the reports on SeqA localization. Unfortunately the cell cycle parameters were not determined and the authors could not make any conclusions as to whether the replication forks were separating or if they were looking at separate replication factories at either the middle or the quarters.

In another study the cell cycle parameters were in fact determined (Molina and Skarstad, 2004). The general result was that too few foci were seen to support either the separating fork or replication factory model. In stead the authors proposed a model with central replication super factories with as many as 8 to 12 polymerases equivalent to 4-6 forks or 2-3 ‘normal’ factories. Although that is not necessarily incorrect, the model is based on these results only and has not been confirmed by other experiments.

Koppes and co-workers used electron-microscopic autoradiography of cells pulse labeled with <sup>3</sup>H-thymine to visualize the position of newly replicated DNA (Koppes *et al.*, 1999). This was, in contrast to the experiments described above, done in an *E.coli* B/r strain. The cells were grown slowly with a very long B-period and replicating cells had

only two origins and two replication forks (as mentioned earlier B/r strains have shorter D-periods and longer B-periods than K-12 strains). It was shown that newly replicated DNA is mainly found in the middle of the cell around the time of initiation. Halfway through the C-period the distribution of newly replicated DNA becomes very broad and in the end it is clearly off-center. From the results the authors conclude that most or perhaps all replication takes place at the cell center. The observation of broad distributions halfway through the C-period and onwards is justified by the presence of more than one class of cells; some replicating the terminus in the middle and others with replicated and separated termini. This explanation is plausible for the cells close to termination and division but does not satisfyingly explain why the distribution broadens already halfway through the C-period. With the introduction of the Home and Away Segregation Model, the observations can easily be explained. The pulse duration was quite long, 10 minutes, so it is possible that the initial sister loci cohesion has been overcome at the time of sample acquisition. This assumption is supported by the fact that the D-period of these B/r cells were only 12 minutes, hence the period of sister loci cohesion is probably less than that. If newly replicated DNA loci in cells in mid-C-period have escaped sister loci cohesion they will according to the Home and Away Segregation Model end up with one copy at the position of replication, that would be off-center for intermediate loci replicated in the mid-C-period, and the other locus would end up just across the cell center. That goes for the other fork too, resulting in newly replicated DNA being present at the quarters and the center. Add some cell to cell variability of the position of the forks and the replicated markers, and you end up with a quite broad distribution of newly replicated DNA in the cells; exactly as reported by Koppes and colleagues.

#### 1.4.4 Replication forks can separate

As described a lot of work has been done on determining the position and dynamics of the replication forks. There is broad agreement on the position of forks; separated or not they tend to localize to mid-cell or the quarters. When there are more forks, the pattern is less obvious and poorly investigated. The reports on the number of forks are more divergent for all growth rates and very often not correlated to the cell cycle making interpretations on fork associations impossible.

Two experiments using synchronized cells with only one replicating chromosome have shown that initiation occurs in the middle and that forks migrate in opposite directions towards the quarter position by visualizing forks directly (Bates and Kleckner, 2005) or indirectly (Adachi *et al.*, 2006). These results are very convincing if not definitive, leaving us with no doubt that forks can separate during replication. The question is if that is normal

or only occurs at low growth rates. The results are contradicted by those of Molina et al.. These results are for faster growing cells though, which might possibly explain the difference. According to Bates and Kleckner the forks are very dynamic once separated, and not located at a fixed quarter positions. That would fit well with the idea of forks tracking along the DNA. This study is one of very few where the actual polymerase has been labeled and should thus be weighted accordingly. Adachi et al. do not describe the localization pattern of the SeqA foci in their study.

In this thesis focus is on the simple cell cycle with just one replicating chromosome. Under these conditions it is not only possible but very likely that replication forks separate.

## **1.5 Dynamics and organization of the replicating chromosome**

The development of these techniques for visualizing DNA loci in bacteria has accelerated the research in chromosome dynamics tremendously. The following is a mini-review of most of the important discoveries including the results from this work. Focus is on the location of chromosomal markers in the cell. The relation of this information to the dynamics of the replication forks will be summed up in the discussion.

### **1.5.1 The origin**

The pioneering work of Gordon and co workers in 1997 showed that the origins are mainly located very close to the cell poles (Gordon *et al.*, 1997). This was the first paper where DNA loci had been visualized in living cells of *E.coli*, and they used the *lacO*/LacI-GFP system. There were however several factors in the study that made the results questionable. First of all, although the experiments were ground-breaking and original for the time, the quality of the pictures were not very good compared to later published results from similar experiments. It is doubtful that they were able to recognize all foci in all cells. But more importantly the authors did not know the cell cycle parameters. Hence they did not know how many origins to expect. The fact is that they only observed cells with either 2 or 4 foci but the cells likely had at least from 4 to 8 origins (Michelsen *et al.*, 2003). Perhaps the cells were sick or in another way affected by the labeling system used, in which case the value of the observations are dubious. Supporting that the cells were affected and not growing normally is the size distribution of the cells. They were grown in LB and thus expected to be quite large, but reported to be in the range of 1-2  $\mu\text{m}$ , which is a lot smaller than normal (Begg and Donachie, 1978). Except from one study from the same lab (Gordon *et al.*, 2002), the result that the origins are located at the cell poles were never reproduced. No other group has published similar results for *E.coli*. It has later been shown

repeatedly that the origins normally are located in the middle, at the quarters or at the 1/8, 3/8, 5/8 and 7/8 positions of the cell depending on the number of loci in the cell (Lau *et al.*, 2003; Li *et al.*, 2002; Niki *et al.*, 2000; Niki and Hiraga, 1998)

Another of the very first experiments where the location of origin and terminus was determined in growing cells of *E. coli* used FISH of fixed cells (Niki and Hiraga, 1998). In this study the origin was found to be located in one end of the nucleoid at cell birth and the terminus in the opposite end. The terminus was very near the cell pole whereas the origin was somewhat closer to the quarter position due to the asymmetric position of nucleoids in newborn *E. coli* cells. They further found that the origin appeared to duplicate at the quarter position where after one stayed and the other went to the other quarter. This is in contrast to later publications where the origin usually duplicates at mid-cell. The terminus migrated to mid-cell around the time where the origin duplicated. The authors verified the results in a later publication where they also showed that only the cells growing at a moderate growth rate (52-55 min doubling time) had the origin towards the old pole when newborn (Niki *et al.*, 2000). At lower growth rates the cells were born with the origin at mid-cell. Apparently it localized to mid-cell before initiation of replication. Presumably the faster growing cells did not 'have time to' put the origin at mid-cell prior to initiation. The results by Niki and Hiraga are quite convincing. They do not determine the cell cycle parameters however, but nor do they claim to present evidence for any of the chromosome segregation models.

Later publications on the position of the origin have in general confirmed the above observations (Bates and Kleckner, 2005; Lau *et al.*, 2003; Li *et al.*, 2002; Nielsen *et al.*, 2006a; Wang *et al.*, 2005). Li and coworkers showed that origin labeled with the GFP-*parB/parS* system separated after initiation with a delay of 1/5 of the generation time and localized to the cell quarters in cells growing in minimal glucose media supplemented with low levels of casaminoacids (Li *et al.*, 2002). Even at faster growth rates the origins are at the quarters when present in two copies (Lau *et al.*, 2003). These cells end up with 4 origins at the 1/8, 3/8, 5/8 and 7/8 positions. The off center positioning of the origin towards the new pole at cell birth has been reproduced at very slow growth rates (Bates and Kleckner, 2005). The origin will under these conditions migrate to the cell center before it is duplicated.

The consensus is thus that in slow growing cells the cell has one origin at the cell center at cell birth or soon after. This origin duplicates and migrates to the quarters, perhaps all the way to the poles of the nucleoid. When it divides the cell once again has one origin at or close to the center. Less evidence exists for faster growing cells, but it seems

that the pattern is very much the same, i.e. they start out having 2 origins, one at each quarter that duplicates and end up at the 1/8, 3/7, 5/8 and 7/8 positions

### **1.5.2 The Terminus**

#### **Pre-division cells**

The terminus is without doubt located in the center of the cell before cell division (Bates and Kleckner, 2005; Gordon *et al.*, 1997; Lau *et al.*, 2003; Li *et al.*, 2002; Li *et al.*, 2003; Nielsen *et al.*, 2006; Niki and Hiraga, 1998). The terminus also duplicates at mid-cell and the two sister copies stay there until and usually beyond cell division (Nielsen *et al.*, 2006; Niki *et al.*, 2000; Niki and Hiraga, 1998). FtsK is believed to play a role in positioning the terminus in the middle, supported by the results of Li and coworkers where they showed that an *ftsK* C-terminal deletion mutant often did not position the terminus properly (Li *et al.*, 2003).

Lau *et al.* reported that the terminus is asymmetrically positioned relative to the middle of the cell, very often being on the new pole proximate side of the center (Lau *et al.*, 2003). They showed that the FtsZ-ring assembles at the middle/quarter prior to the migration of the terminus from the pole/middle, and that the terminus stays on the inner side of the FtsZ-ring.

#### **Post-division cells**

The terminus is found close to the new pole in newborn cells (Niki and Hiraga, 1998) and, depending on the growth rate, it migrates to mid-cell soon after cell division. It appears that at faster growth rates this migration happens almost instantly after division (Li *et al.*, 2003; Niki and Hiraga, 1998) whereas in slower growing cells the terminus can stay at the end of the nucleoid for as much as half a generation (Li *et al.*, 2003).

This migration, often referred to as a ‘jump’ as it is a very rapid movement, has been reported to coincide with another jump in the cell in a FISH experiment using slowly growing synchronized *E.coli* cells (Bates and Kleckner, 2005). The duplicated origin copy that resided in the same half of the cell as the terminus (i.e. is the new pole half) was found to locate between the quarter and mid-cell soon after duplication of the origin foci. At the same time the other origin localized to the quarter in the other half of the cell and the terminus was still at the pole. When the terminus ‘jumped’ to the middle of the cell, the origin in the same half of the cell made a smaller jump to the quarter position where after the two origins were positioned symmetrically at the quarters and the terminus in the middle.

### Assymmetric distribution of terminus proximal markers

Wang et al. found that terminus proximal markers were very often positioned asymmetrically. A locus 200 kb left of the *dif* site was frequently found close to the pole in one end of the cell and a locus 200 kb to the right of *dif* in the other end close to the other pole (Wang *et al.*, 2005). Both these sites are in the terminus region and fairly close to the *dif* site, and they separate very late in the cell cycle. It was surprising to find that two loci positioned only 400 kb from each other are located in each end of the cell. Naturally the DNA in between, containing the *dif* site, has to span the nucleoid from one end to the other. In time-lapse they further showed that these loci migrated from the pole to the middle at the time of replication and then one stayed in the middle and the other returned to the pole it came from.

Based on this discovery Wang et al. (2005) proposed a model where the entire left arm of the chromosome is in the left half of the cell and the entire right chromosomal arm is in the right half with the origin and terminus in the middle connecting the two halves. Following initiation the origins migrate to the quarter positions and the replicated left chromosomal arm goes to the outside of the left origin and on the inside of the right origin where as the right chromosomal arm goes to the outside of the right origin and to the inside of the left origin after duplication. This way of organizing the DNA would ensure that the origin and terminus ends up in at the quarter; or middle of the coming daughter cell. Wang et al. further proposed that (for example) the leading strand would always end up on the outside and the lagging strand on the inside. This model is basically a slightly less detailed version of the Home and Away Segregation Model.

### 1.5.3 Intermediate markers

There has not been done nearly as much work on the position and dynamics of the DNA between the origin and the terminus as there has for these two loci in particular. It is understandable that the position of the origin and terminus are the first to be investigated, as these were expected to give away the most information. That assumption is possibly incorrect because these two loci are located very symmetrically in the cell. The localization patterns of the origin and terminus are quite trivial whereas intermediate loci have turned out to show more complex and very asymmetric localization patterns. This has very recently led to insight into the general organization of the entire chromosome as described in the following.

At slow growth rates the chromosome is organized with one arm in one half of the cell and the other arm in the other half. The first results indicating this organization was published 6 years ago by Niki and coworkers. They showed that the cell is born with the

origin and terminus at mid-cell, the 70' locus in one end of the cell, and the 20' locus in the other end at slow growth (Niki *et al.*, 2000). This was a minor result of the paper and was not given much attention. Recently similar results have been produced (Nielsen *et al.*, 2006; Wang *et al.*, 2006). With the origin and terminus in the middle of the cell and the two arms in opposite sides, it seems that the chromosome is actually organized as a condensed ring inside the cell; at least at slow growth rates.

#### 1.5.4 The centromere like sequence: *migS*

If there is a specific sequence responsible for the localization of the origin it has not been identified. Such a sequence would have to be located outside the minimal *oriC* sequence as it has been shown that *OriC* plasmids carrying the minimal *oriC* region do not localize in the cell, and are easily lost without an additional partitioning system as for example the *sopABC* present on the plasmid (Niki and Hiraga, 1999).

In a search for a sequence responsible for the positioning of the origins, Yamaichi and Niki identified a 25 bp sequence in the *yijF* open reading frame located at 89 min on the chromosomal map, 5 min clockwise to the origin (Yamaichi and Niki, 2004). Without this sequence, named *migS* (for Migration Site), origins tend to be much closer together in cells with two separated origins. Other than that it does not have any significant effect on the cell cycle and growth rate.

Fekete and Chattoraj have further shown that cells segregate the 89' locus earlier than the origin and segregate this locus further out (Fekete and Chattoraj, 2005). However without the *migS* sequence this locus segregates in line with the origin. These results too indicate a role for *migS* in origin segregation, but are in direct contrast to our own results (paper II). We usually find that the 89' region is delayed in its segregation.

So far no other function has been found for this seemingly redundant site, and its role and importance in the chromosome segregation remains elusive.

#### 1.5.5 The timing of segregation

There has not been put much effort into the determination of the timing of the segregation. That may be surprising as this is quite important and could reveal a lot about which of the segregation models is closer to the truth.

Of course loci can not segregate before they are actually replicated. Either they segregate right after replication or some time after. Exact knowledge of the replication cycle is therefore imperative in order to learn anything about the timing of segregation.

Bates and Kleckner did an attempt to relate the timing of segregation to the replication. They used synchronous cells of known replication cycle and found that markers segregated

some time after replication, thus supporting a Sister Cohesion Model. Unfortunately they only looked at 2 markers beside the origin and terminus. Hence their conclusion that what they see is Sister Chromosome Cohesion is doubtful. That kind of cohesion would mean a longer delay between replication and segregation at earlier markers and a shorter delay for later markers. We do not think that 2 markers outside the origin/terminus region are enough to determine if there are such differences in the replication-segregation delay. Furthermore the C and D periods determined for the strain they used are very different from what is normally seen for that strain (F.G. Hansen, personal communication), so possibly the timing of replication was not known in the study by Bates and Kleckner (cooper microcommentary ref ??).

In our first paper we have looked at the timing of segregation of 14 markers (Nielsen *et al.*, 2006a). We used 14 different strains with known cell cycle parameters. This study too revealed that there is a delay between replication and segregation. Due to the high number of loci examined we were able to determine that this segregation delay is constant for all markers, thus supporting the Sister Loci Cohesion model and not the sister Chromosome Cohesion model. In fact this study was the one that lead to the Sister Loci Cohesion Model. This segregation pattern was also seen at higher growth rates with continuous replication (see section 2.4). Hence it is clear that the chromosome is segregated progressively at slow and moderate growth rates.



## **2 Results**

In the following the most important results of my 3 year study is presented. The major results are also described in the accompanying papers (I, II, and III). The results are presented more or less in the chronological order in which they were obtained. Some of the results have already been mentioned in the introduction and some of them will be discussed next in the discussion. However I feel it necessary to explain exactly what I have been doing in this three year Ph.D. project, including some of the minor and/or preliminary results.

### **2.1 Counting and measuring cells**

When I began this work most chromosome labeling studies were focused on labeling the origin and terminus of replication and determining the localization patterns of these markers. I wanted to expand the number of sites to cover the entire chromosome in order to get a better and more detailed picture of the chromosome organization, and I also wanted to investigate the localization of these markers in the cell at many different growth rates with varying number of chromosome equivalents optionally adding different mutations to the strains too. The main challenge in order to do this was not the creation of the strains nor growing them and taking pictures, but rather the very time consuming job of measuring the cells and counting the foci within.

The minimum number of cells that needs to be counted for a given experiment (one marker at one set of growth conditions) is around 1000 to get decent results but preferably 1500 or more. Using manual measuring a dataset of 1000 cells takes in the range of 3-5 hours. Hence a dataset consisting of 14 different markers as the one published in the accompanying paper (II) would take around 60 hours; and 90 hours or more if 1500 cells per marker are to be measured. That is just for one set of growth conditions. If one wants to do different growth rates and/or specific mutations too the amount of time spent counting and measuring cells is endless.

So I decided to develop a fully automatic computerized method for counting and measuring cells and their foci. This was my first objective of this study. Instead of creating a new program from nothing I decided to use one of the advanced digital image analysis

programs that allow programming of macros connecting the relevant functions of the software. I chose Image Pro Plus which is a very useful and versatile program containing programmable features that can outline, count and measure objects saving me a lot of time that I would otherwise have to use programming these functions myself.

A description of the macro I made is found in the accompanying paper (I). It is capable of automatically identifying the cells from phase contrast images and measuring the length and width of them. Subsequently it goes through the fluorescence signal from each cell individually counting and measuring the foci within. The algorithms and procedures used for finding the foci are very effective identifying more than 99% of all foci. Closely located pairs of foci are resolved using a separate procedure. The macro also inspects cells for invagination of the cell membrane and gives invaginated cells a score reflecting the degree of invagination. This is very useful for isolating the dividing cells in the resulting dataset and analyzing these separately.

The macro was validated by comparing the result of counting the same dataset manually and automatically. As presented in the accompanying paper (I), the macro does a much better job at measuring cells consistently than by measuring by hand. Not surprisingly manual counting is best for identifying the foci, but nevertheless the macro identifies 99% of them. Furthermore the macro is more consistent in deciding whether or not very faint and doubtful foci should be counted or not where this decision depends very much on the person doing the inspection when measuring cells manually. Most importantly of course is the speed of the process. The macro counts 1000 cells in 3-5 minutes depending on the quality of the pictures, whereas manual counting of the same number of cells takes 3-5 hours. Hence 14 different datasets (for 14 different markers) of 1500 cells each is done in an hour or so; a job that would take weeks by manual counting.

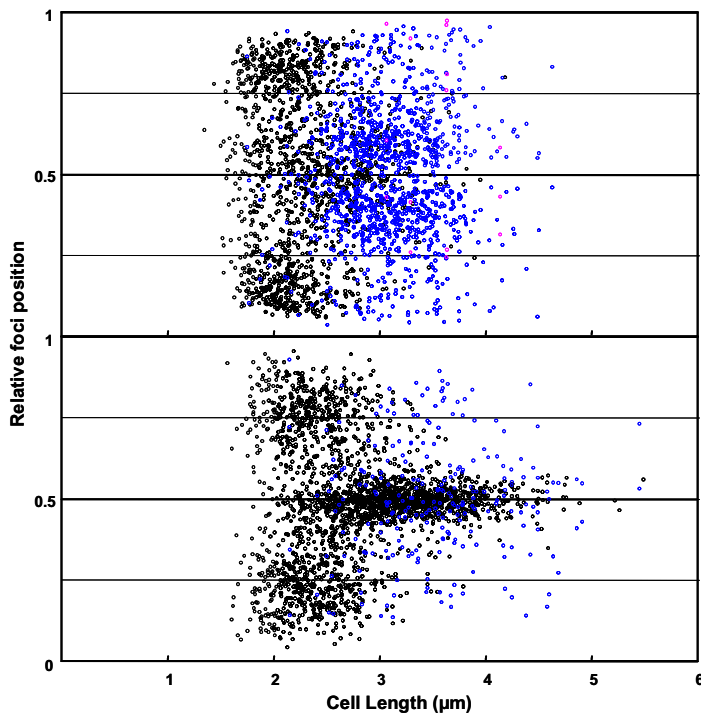
## 2.2 Optimizing the P1-par labeling system

The labeling system used to map the intracellular position of markers in this study is the GFP-P1-ParB/*parS* system developed by Li et al. (Li *et al.*, 2002). The original procedure as they reported it was to induce the GFP-ParB from the upstream *lac* promoter at 100  $\mu$ M IPTG for three hours before sampling. I wanted to change this pulse induction to a lower and continuous induction, first of all because we did not know how the high induction affects the balanced growth of the cells, secondly because we were experimenting with growing the cells in a chemostat where continuous induction of course is desirable, and finally because continuous induction is better suited for long term time-lapse studies.

While experimenting with the best choice of induction level I realized that the total number of foci observed was much higher when inducing continuously with 20  $\mu$ M IPTG or lower compared to the 100  $\mu$ M pulse induction. It turned out that the 100  $\mu$ M pulse induction made very large foci often containing 2 or more of the labeled loci. As presented in the accompanying paper (II) the observed number of foci in the cells got very close to the actual number of loci present when induction was absent (0  $\mu$ M IPTG) relying only on the basal expression from the *lac* promoter. We already knew this was not the case when pulse inducing with 100  $\mu$ M IPTG (compare the results in Nielsen et al. (2006) with Li et al. (2000)). Thus further experiments were carried out without IPTG induction.

The presence of fewer foci is not a result of fewer loci or less DNA in the cells in general. That would be very unlikely as the growth rate of the cells is not affected by the induction. Cells can grow at full (>100  $\mu$ M IPTG) induction continuously at normal growth rate. Furthermore flow cytometry analysis showed similar DNA/mass ratios in the induced and non-induced cells. Therefore the only possible explanation is that two or more loci stick together in single foci.

This conclusion was strengthened when looking at the patterns of foci localization, which are very different between the two induction strategies. As seen on Figure 2.1, the high level short term induction creates fewer foci (less blue dots in the figure). In the larger cells where two foci are expected very often only one focus is observed in the center of the cell. This turned out to be a general phenomenon: When two or more loci stick together in one focus at high GFP-ParB concentrations the focus locates to the cell center (or



**Figure 2.1 Stickiness.**

The number and pattern of observed foci are shown under conditions with and without stickiness for the same strain. A strain expressing GFP-P1-ParB with a *parS* site inserted at 21' on the chromosomal map was grown in the absence of IPTG (upper panel) or with 100  $\mu$ M IPTG (lower panel) for 6 generations in ABT glycerol minimal media at 32 degrees. The number of foci and their relative position on the long axis is shown as function of cell size. Cells containing one focus are represented with a black dot and cells with two foci with blue dots. 2000 cells are plotted in each panel.

alternatively the cell quarters for higher growth rates, data not shown). We call this phenomenon ‘stickiness’. Stickiness is an interesting effect in itself but unwanted when investigating the normal position and segregation of markers. Therefore chromosomal labeling studies were done under conditions without stickiness. An investigation on stickiness has begun and the preliminary results is presented in section 2.7.

## **2.3 Time-lapse studies and flowcells**

In parallel to the still-image analysis I have experimented a lot with time-lapse studies. In a time-lapse study a few cells sitting on the slide is followed over time by photographing them at regular intervals. Time-lapse has the advantage that it allows us to track the movement of specific foci inside the cells directly. It is not necessary to infer the dynamics of the foci from statistical analysis of a lot of snap shot images. A major disadvantage though is that it is time consuming and very few cells can be analysed at a time limited by the small size of the field.

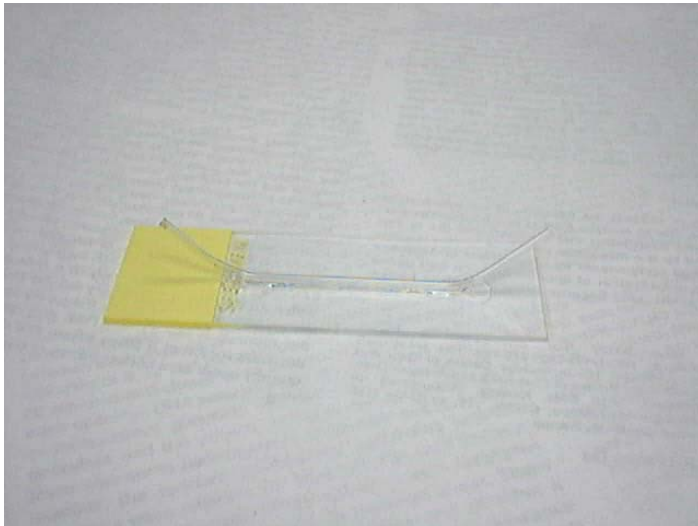
Most time-lapse studies in the field of chromosome dynamics are done on agarose slabs. Cells are grown to balanced state in a liquid culture and then transferred to a 1-2 mm thick agar slab on a microscope slide and covered with a cover glass. The slab contains the same media as was in the liquid culture and cells will thus continue to grow on the slide, for a while at least. The slide is mounted under the objective and the growth of the cells monitored.

There are several major problems with this technique. First of all and most importantly the cells will get out of balanced growth soon after they are put on the slab because the oxygen concentration in the slab will drop and quickly become limiting. The nutrients will last longer but will eventually run out too. The temperature is another problem but can be controlled by heated stages. The result is that cells often only grow on the slide for a few generations (depending on the media) and then stop.

A few generations are not enough to do extensive studies on the dynamics of foci inside the cells. Furthermore, because the cells are not balanced, the cell cycle parameters will not be known and the relation between replication and movement of foci in the cell can not be established. Therefore I decided to experiment with doing continuous time-lapse of cells growing on a fixed surface with fresh media constantly flowing past the cells providing balanced growth conditions and cell growth for many generations.

### 2.3.1 Designing a flowcell

In biofilm research the use of flowcells is widespread. A flowcell is a chamber with a tube going in and out of it and a glass slide on one side functioning as cover slide so that the flowcell can be mounted on a microscope. Fresh media is lead into the chamber through the tubing and cells grown on the inside of the glass surface. Unfortunately the commercially available flowcells are unsuitable for phase contrast microscopy. Commercially flowcells are designed for confocal or differential interference contrast microscopy. They are too thick to do phase contrast or brightfield microscopy because the condenser collector lens can not get close enough to the focal plane. Furthermore they only have one glass side; the bottom is made of plastic with a refractive index incompatible with a phase contrast or brightfield microscopy setup.



**Figure 2.2 Flowcell construct.**

The flowcell used for time-lapse studies. An 8 cm capillary glass tube was heated 1 cm from each end and bend to a 45 degree angle. The glass tube was then glued to a glass slide at the ends and immersion oil was put between the tube and the glass slide at the middle. Rubber tubing was mounted at the ends of the glass tube and everything disinfected before balanced cells were injected into the flowcell using a syringe and needle. The flowcell was then mounted on the microscope and fresh media was pumped though it continuously.

So I had to design my own flow cell that was thinner and made entirely of glass. After a few unsuccessful constructs I realized that this was a very simple task. I simply bend the ends of a capillary glass tube and glued it to a glass cover slide (Figure 2.2). The cells grew on the inside of the glass tube and fresh media could be pumped through the rubber tubing mounted on the bend ends of the glass tube. Immersion oil was applied directly to the glass tube and the whole thing mounted on the microscope stage. Cells were injected through the mounted rubber tubes with a syringe and needle. The capillary glass tube was coated on the inside with poly-L-lysine before being glued on so that the injected cells would attach to the glass surface. After injecting the cells the pump was turned on pumping fresh media through the tube. The temperature was kept constant at 32 degrees in the microscopy room to keep the media from cooling down the stage.

This construct worked great and allowed the monitoring of cells for many generations.

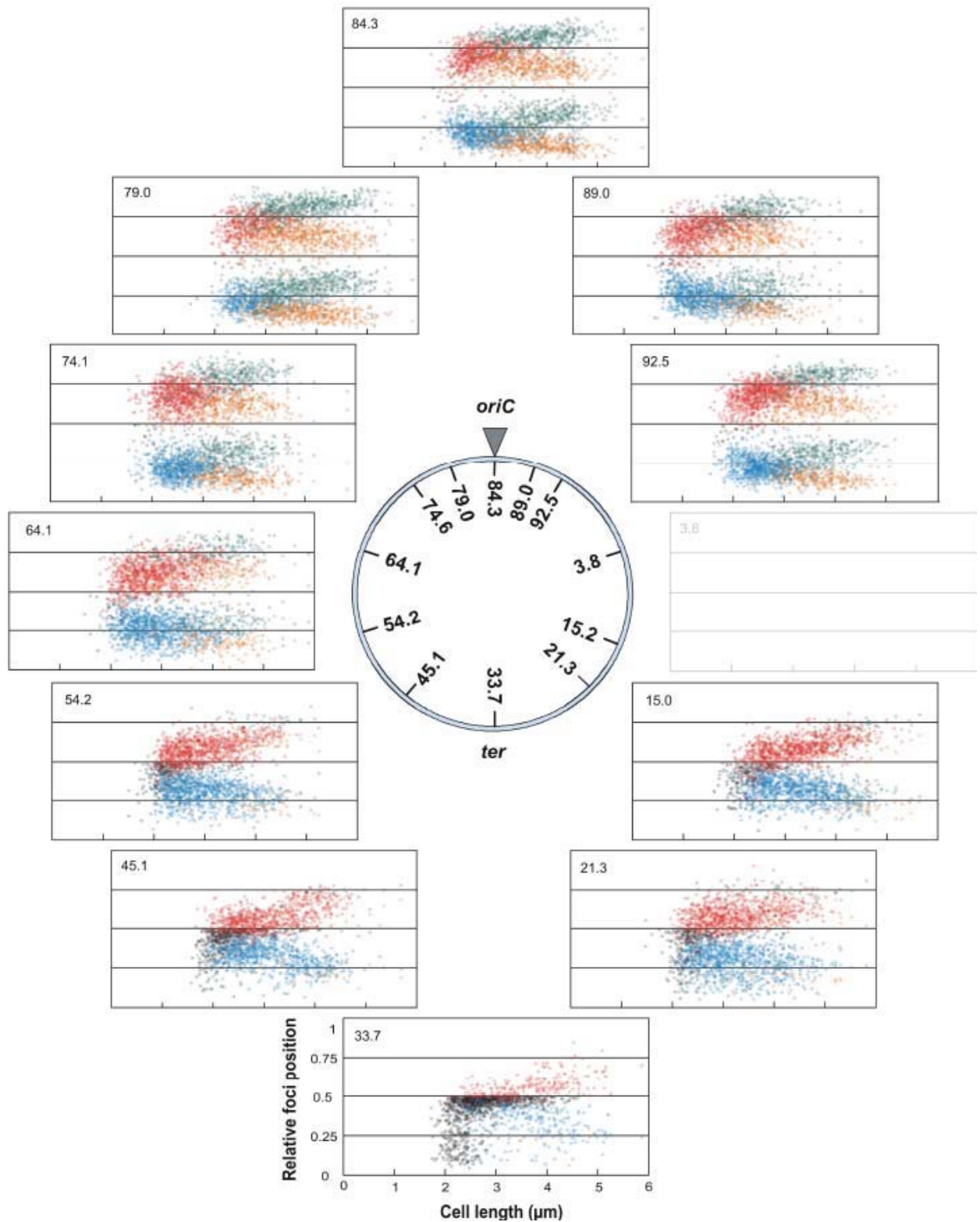
### 2.3.2 Short term time-lapse

Unfortunately it turned out that time-lapse was not all that great after all. While just looking at the cells growing in the flowcell I observed that foci were very mobile and moved a lot inside the cells. Therefore I did some very short term time-lapse experiments. It turned out that foci are so mobile inside the cell that the position of a focus can change as much as half a micron in seconds (see paper II). Hence when doing time-lapse experiments with e.g. 5 minutes between the images the significance of the recorded position of the foci is very low because they might very well have been located elsewhere just seconds before or after. With this discovery the need of a good statistical basis and a large number of cells increases dramatically and the value of time-lapse experiments becomes dubious. Therefore we decided to stop time-lapse experiments and switch entirely to statistical still image analysis of large number of cells.

## 2.4 Progressive chromosome segregation

Using the previously described macro I analyzed 14 strains with a *parS* insertion at 14 different chromosomal positions. The insertion coordinates were 4', 15', 21', 28', 33', 41', 45', 54', 64', 74', 79', 84', 89', and 93'. The strains were created by Young Fang Li at Dr. Austin's laboratory. I grew the strains in ABT glycerol minimal media to get a DNA replication cycle without overlapping C and D periods, thus keeping the chromosome segregation pattern as simple as possible. The results clearly showed that the chromosome is segregated progressively and also gave clues to the general organization of the chromosome as described in the accompanying paper (II). Furthermore it was shown that there is a constant delay between replication and segregation of markers. This is what we call sister loci cohesion.

The result of a similar series of experiments done in ABT glucose minimal media supplemented with 0.05% casaminoacids is shown in Figure 2.3. These results have not been published. There are clear similarities between the distributions of loci under the two different growth conditions. The origin and its proximal markers tend to locate further towards the pole than later replicated markers for example. The asymmetry of intermediate markers is also evident, although perhaps to a lesser extent.

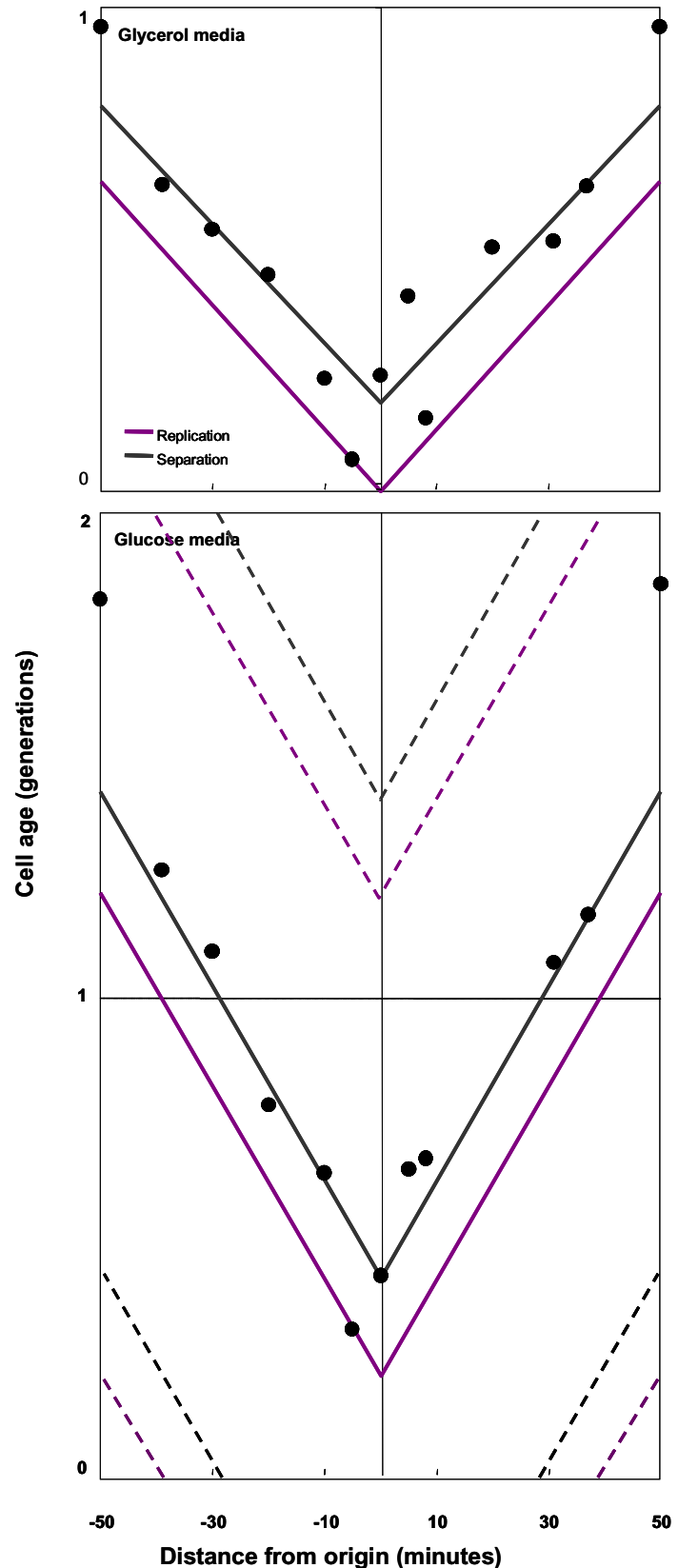


**Figure 2.3 Positions of chromosomal loci in cells growing in glucose media.**

The relative positions of foci are presented for cells with the P1 *parS* at the indicated positions on the chromosomal map (central panel). Foci from cells with one focus are represented by black dots showing the distance to the nearest pole, foci from cells with two foci are represented with a blue dot (the focus which is closest to a pole) and a red dot. Foci from cells with three or four foci are shown with orange dots (the foci nearest to a pole) and green dots. All cells are oriented so that the sum of distances from the pole to each focus in every cell is lowest. In all panels data from 1400 cells are presented. The strain labeled at 3.8' grew poorly in the glucose media and data from this strain is therefore omitted.

One major difference though is the much more compact distribution of markers in the cells growing in the richer media. The markers seem to be constrained to smaller and better defined areas of the cell at the higher growth rate. This could be a result of more DNA content of these cells that have a higher DNA replication activity and a need for better organization of this DNA.

Flow cytometry analysis showed that the cells have a C period of 55 minutes and a D period of 43 minutes at a generation time of 55 minutes. Thus DNA replication is initiated 12 minutes after cell division at the same time the previous round of replication is terminated and cells have from 2 to 4 origins. The timing of segregation compared to replication for the different markers is shown in Figure 2.4. As with the glycerol cells a progressive segregation is observed. Furthermore the delay between replication and segregation (sister



**Figure 2.4 Progressive segregation.**

The timing of segregation is shown for cells grown in glycerol (upper panel) and glucose (lower panel). The purple line indicates the time of replication of the chromosomal markers according to the distance from the origin (x-axis) as found by flow cytometry. The dots are the average time of segregation for the markers investigated. For the glucose experiment, loci going from two to four copies are plotted in the lower part (the first generation) and loci going from one to two copies are plotted in the upper part (second generation). The black line is the segregation trendline. Outliers as the 79' and 33' markers and for glycerol the 89' marker too do not contribute to the trendline. The hatched lines represent the previous and following rounds of replication and segregation.



loci cohesion) seen in the glycerol experiment is also reproduced. The average delay was 0.17 generations (20 minutes) in the glycerol experiment and 0.22 generations (12 minutes) in the glucose experiment. These values are quite comparable which may reflect the nature of this sister loci cohesion. Exceptions to the general delay are the marker at the terminus and the 79' locus. The exception at 89' in glycerol behaves normal in the glucose experiment, whereas the 79' locus segregates early in both experiments. The terminus segregates very late as expected of this region.

Combining these results show that the chromosome is progressively segregated both at slow and moderate growth rates and that there is a delay between replication and segregation of about 20% of the cell cycle. It is very difficult to claim chromosome cohesion for extensive parts of the chromosome in the light of these results and the sister chromosome cohesion model can therefore not be correct.

## **2.5 Developing a pMT1-*par* labeling system**

A major disadvantage of the P1-*par* labeling system compared to others is that it only allows labeling of one marker or alternatively more markers but all with the same color. When we observed the asymmetric nature of the localization of intermediate markers in the cell (Paper II) we realized that it would be very useful to be able to record the position of one locus with respect to another. Hence we needed an additional labeling system with its own specificity and its own color.

For this we cloned the partitioning system of the pMT1 virulence plasmid from *Yersenia pestis*. This system is very similar to the partitioning system of the P1 plasmid. It consists of the same three elements, the *parA* and *parB* genes and a *parS* site. It has been shown that the pMT1 and P1 partitioning systems are functionally compatible meaning that the presence of one of them in the cell does not affect the function of the other (Youngren *et al.*, 2000). Furthermore substituting the ParB in one system with the homologue protein from the other system, or substituting both the ParB and ParA, results in non functional partitioning. These results suggest that the ParB protein of the pMT1 system does not bind the P1-*parS* sequence and vice versa, and a pMT1-*par* based labeling system is therefore expected to be compatible with the P1-*par* based system.

We constructed a GFP-pMT1-*parB* fusion protein and expressed it in a strain containing a pMT1-*parS* site. We deleted the 23 N-terminal amino acids of ParB similar to the 30 amino acids deletion in the GFP-P1-ParB fusion protein which makes up the ParA binding domain. The pMT1 fusion protein was able to form foci in the pMT1-*parS* containing strain. Furthermore when expressed in a P1-*parS* containing strain no foci were

formed. No foci were observed when expressing the GFP-P1-ParB protein in a pMT1-*parS* strain either. Hence the GFP-ParB fusion protein of one system does not bind the *parS* site of the other. The possibility that the ParB proteins could bind to each other had to be investigated too. The binding of ParB protein to the cognate *parS* site consists of an initial binding of one ParB dimer to the *parS* site and subsequent binding of additional ParB proteins to the first ParB protein along the DNA creating a large linear protein-DNA complex. If the ParB protein of one system is able to bind the ParB proteins of the other, the presence of both in the same cell could lead to mixed binding of both proteins to the same *parS* site even though the ParB proteins are specific with respect to initial *parS* binding. We investigated this by expressing a CFP-P1-ParB and a yGFP<sup>1</sup>-pMT1-ParB in the same cell containing either the P1-*parS* site or the pMT1-*parS* site or both. When only one type of the *parS* site was present the foci formed were of the color corresponding to the cognate fusion protein. When both types of the *parS* were present distinguishable foci of the two different colors was observed. If the ParB proteins were able to bind each other the foci would be of mixed color. As this was not observed we concluded that the two labeling systems are completely compatible.

See supplementary material of Paper III for details on the construction of the pMT1-*par* labeling system.

## 2.6 Separate replichores localize to separate cell halves

Having developed and validated an additional labeling system the next step was to look at cells labeled at two different sites. Several different combinations are potentially interesting, but in the light of the results of Paper II we chose to look at markers on opposite sides of the origin. In Paper II we show that intermediate markers on both sides of the origin are very asymmetrically positioned in the cell; either one focus off-center or in the presence of two, one focus close to the pole and the other at the cell center. The question of course was then if the intermediate markers on opposite sides of the origin-terminus axis would then localize to different ends of the cell, or if their localization was independent of each other.

It turned out that loci on one arm of the chromosome are indeed localized to one half of the cell and loci on the other half is located in the other half of the cell. This result is described in Paper III. When we labeled loci on the same chromosomal arm the

---

<sup>1</sup> yGFP is a GFP mut2 variant with a red-shifted excitation spectrum and a normal emission spectrum. The red.shift makes it suitable for use together with CFP. See supplementary material of Paper III for details.

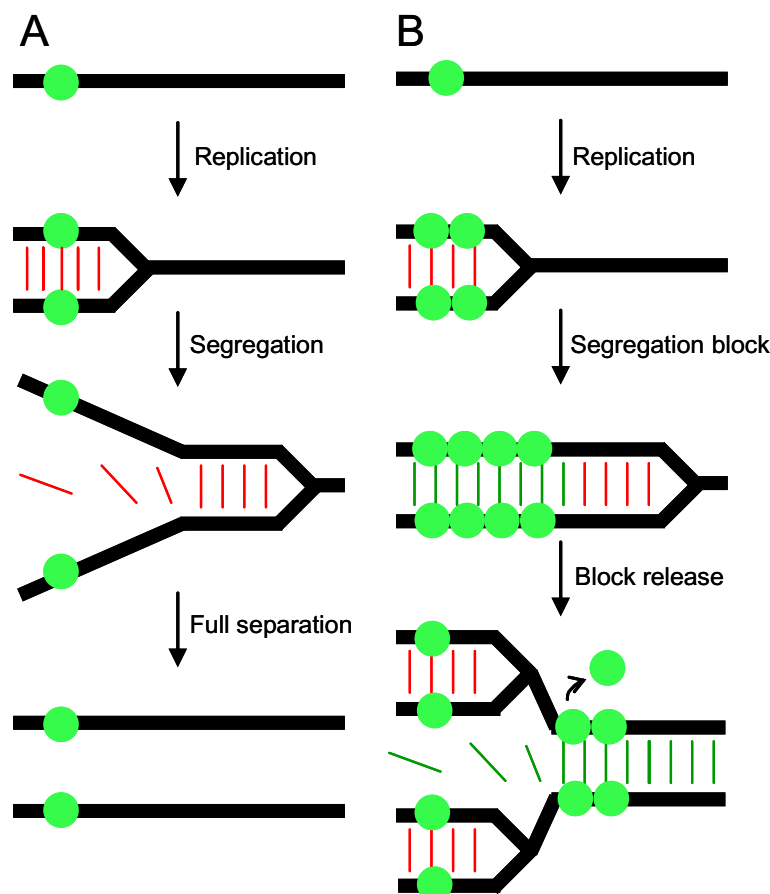
colocalized to the same cell half, and when labeling loci on opposite chromosomal arms they localized to separate cell halves. After duplication when 2 of each locus are present in the cell they often localized on separate sides of the quarters with a strong bias towards a tandem configuration. That is a left-right-left-right chromosome arms configuration.

At the same time our paper was accepted Wang and coworkers published a similar paper presenting basically the exact same results as ours (Wang *et al.*, 2006). The conclusion was also the same, that the chromosomal arms are separated to distinct cell halves in the cell. Wang et al. further showed that the original configuration was very often maintained in the tandem configuration so that a left-right orientation results in a left-right-left-right (chromosome arms) configuration and only rarely in a right-left-right-left configuration. This fits with our Home and away Model.

## **2.7 Stickiness**

As described previously we have found that high levels of GFP-P1-ParB cause a reduction in the number of observed foci. We call this phenomenon stickiness because we believe that what we see are pairs of loci sticking together as I will argue in this section. The phenomenon is undesirable in the chromosome labeling studies done as part of this thesis and the experiments have therefore been carried out at levels of GFP-ParB protein where stickiness is not present, but it is an interesting feature of the ParB/*parS* system that could be important in the original function of plasmid partitioning and might also be useful in chromosome dynamics studies. Therefore we have begun an investigation of the phenomenon.

Two possibilities can be imagined to cause a reduction in the observed number of foci. Either there are fewer loci present in the cell or loci stick together. In other terms, either DNA replication or chromosome segregation is delayed or blocked. It is likely that bound ParB protein can block the replication forks and thus cause a reduction in the number of loci. It has recently been showed that the *tet* repressor/operator system is capable of such tight binding (Possoz *et al.*, 2006). To investigate if the replication is blocked under high concentrations of ParB protein we analyzed non-induced and fully induced cells by flow cytometry. The resulting cytograms showed that the DNA to mass ratio was the same for the induced and non-induced cells. Furthermore the growth rates of induced and non-induced cells are the same. Thus initiation and progression of replication occurs normally. If replication is blocked the cells would expect to die when induced as reported for the *tet* system. That does not happen when stickiness is induced; the cells grow fine.



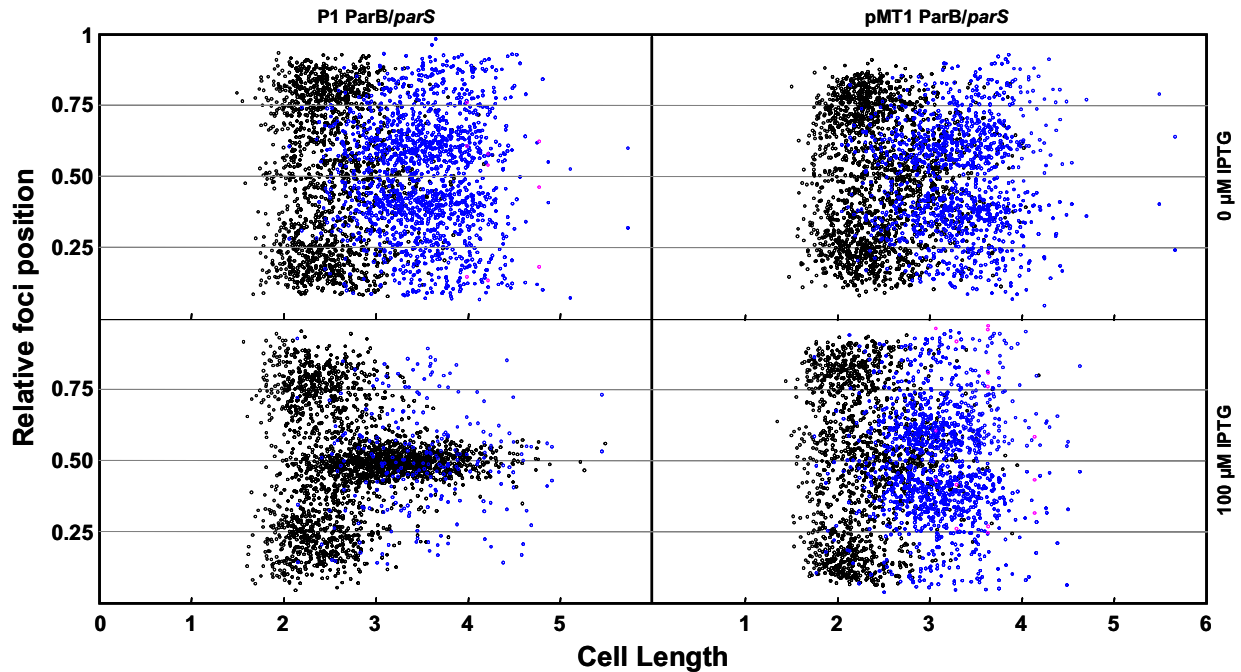
**Figure 2.5 Blocking segregation.**

Normal replication and segregation is shown in (A) and a proposed model for segregation blocking by stickiness in (B). *parS* bound GFP-ParB is shown as green balls. Under normal conditions a limited number of proteins bind the DNA (A) and the two sister strands can segregate normally. When over expressed, too many ParB proteins bind the DNA (B) and makes the two sister strands glue together unable to separate (B; green bars). Finally when the next round of replication reaches the block, the ParB proteins are peeled of and the daughter strands can separate. These daughter strands are now already duplicated and segregate as double duplexes. The red bars symbolizes sister loci cohesion.

The other possibility that segregation is delayed must then be correct. That implies that a specific mechanism responsible for DNA segregation and independent of replication is present, which fits fine with our observation of sister loci cohesion. Why then is blocking of DNA segregation not lethal? One possible explanation is that the segregation block is released when the next round of replication reaches the block. As the bound ParB can not block the replication fork any bound ParB must be peeled of by the replication fork. Hence any ParB blocking segregation bound in the trail of replication will be removed in the next round of replication. Segregation will then segregate two pairs of duplex molecules (Figure 2.5).

The delay of segregation also means that induced cells are larger than normal cells. As mentioned the DNA/mass ratio is the same with and without induction but the absolute DNA content is higher and the cells are bigger when stickiness is induced. As the growth rate and the initiation mass are unaffected the only possible explanation is a prolonged D-period in the induced cells. This has been confirmed by flow cytometry. A prolonged D-period means that cell division is delayed which in this case is probably caused by the delay of segregation.

The stickiness reported here was observed for the P1 *par* system. Surprisingly the very recently developed pMT1 system does not show this effect when induced at high levels. This can be seen in Figure 2.6. The P1 *parS* and pMT1 *parS* sites were inserted at the same



**Figure 2.6 The pMT1 system is not sticky.**

The relative position of foci were measured for two different strains: One with a P1-*parS* insertion at 21.3' and expressing the GFP-ParB protein of P1 (left panels) and another with pMT1-*parS* inserted at 22.1' expressing the GFP-ParB of pMT1 (right panels). The upper panels show the distribution of foci at low ParB concentration (no induction of the *lac* promoter), and the lower panels show the distributions at high concentration of the ParB proteins (full induction, 100μM IPTG). Stickiness is observed only for the strain with a P1-*parS* site expressing GFP-P1-ParB at full induction. The cells were measured using the macro described in Paper I. 2000-2300 cells were counted for each panel.

position in two different strains and the cognate GFP-*parB* protein expressed from a plasmid. At low expression, without adding IPTG, the observed patterns of foci localization in the cells are essentially the same (compare upper panels). At full induction (100 μM IPTG) the sticking together of pairs of foci at the center of the larger cells is easily observed for the strain with P1 GFP-ParB/*parS*. But for the strain with a pMT1 *parS* site the pattern is unchanged at the high induction. Thus the pMT1 GFP-ParB does not cause stickiness. This result supports the findings of Youngren et al. that the pMT1-ParB does not cause silencing of nearby genes like the P1-ParB does (Youngren *et al.*, 2000). In the light of this result the pMT1 system becomes the best choice in future single color studies because the possibility of expressing the GFP-ParB at higher levels gives brighter foci without risking that they stick together. When doing time-lapse studies this system will also be superior; not only to the P1 based labeling system but also to the *tet* and *lac* operator/repressor based system. That is because the problem of bleaching of foci caused by several successive exposures in time-lapse can be compensated by using the pMT1 system at high levels of GFP-ParB. This is not possible with P1-ParB as it will delay

segregation or with the *tet* repressor/operator system as it will cause replication to get blocked.

The fact that high levels of GFP-P1-ParB cause sister loci to stick together might very well be functionally important for the original role of this protein in plasmid partitioning. Many models of *par* mediated plasmid partitioning suggest that pairs of plasmids are held together at the central plane of the cell before they are actively pulled apart by the ParA proteins. The fact that the GFP-ParB of P1 is capable of holding sister *parS* loci together supports such models.

So far stickiness has been an unwanted feature of the GFP-ParB/*parS* labeling system and experiments have been carried out at non-sticky levels of the GFP-ParB protein, but stickiness could potentially be very useful in chromosome dynamics studies. One could imagine an experiment where segregation is blocked at one site by stickiness and the fate of another locus located before or after the blocked site monitored. This other site could then be labeled by the pMT1-*par* labeling system that is not sticky.

### **3 Discussion**

Most relevant published data in the area of chromosome segregation has been presented in the first chapter. It is evident that there are many different opinions on how the chromosomes segregate; perhaps even more opinions than data justifies. Much of the confusion is due to significant divergence on the interpretation of the presented data by various scientists.

The results of my three year ph.d. project has helped clarify some of the major questions in the field. The three major results of this work, as represented by the three accompanying papers, are the development of a fast and reliable automatic method for measuring cells, the finding that chromosome segregation is progressive and finally that the chromosome of slow growing cells is organized with each chromosome arm in separate cell halves. Minor results like the development of an additional labeling system and the finding of sister loci cohesion will hopefully become important in future research in the field. The implications of these results and how they affect our view on chromosome dynamics is discussed in the following.

#### **3.1 A model for chromosome segregation**

A model for chromosome segregation should ideally apply to all growth rates. However as the knowledge on chromosome dynamics is still limited and the cell cycle of *E.coli* can be very complicated we found it a good idea to initially attempt to make a basic model valid for slowly growing cells with a simple cell cycle. Hence we decided to do a thorough study of chromosome dynamics using slowly growing cells with no overlapping replication cycle. In these kinds of cells we could follow the segregation of two replicated chromosomes without the influence of subsequent rounds of replication simplifying the interpretation of the results. The resulting model may not be precise or detailed enough to describe the chromosome dynamics of *E.coli* cells at all growth rates, but will ideally form a basis for future studies on chromosome dynamics of fast growing cells.

### 3.1.1 Progressive segregation

In this thesis I chose to use the process of replication as reference in my effort to determine and describe the process of chromosome segregation. The process of replication is a logical basis since the duplication of the chromosome by replication is an explicit requirement for segregation. This may seem trivial but I find that quite many studies of chromosome segregation fail to relate results to the DNA replication cycle of the cells, which I think is a mistake.

This relationship between the process of replication and the process of segregation can be divided into a temporal relationship and a spatial relationship. That is WHEN the chromosomes are segregated compared to when they are replicated and from WHERE they are segregated compared to where they are replicated. With this information a quite detailed model on chromosome segregation can be established, and certainly the existing models can be evaluated by it. The central factory models for example predict that replication and segregation is coincident in time and space (at the cell center), whereas the Sister Loci Cohesion Model predicts a considerable delay between replication and segregation.

To address the temporal relationship between replication and segregation we analysed 14 strains labeled at 14 different sites evenly spread on the chromosome (Paper II). We determined the timing of separation for all 14 loci by finding the cell size class where half of the cells had one focus and the other half two foci and converting this cell size to cell age. As presented in the accompanying paper for cells growing in glycerol and further presented in section 2.4 for faster growing cells, loci clearly separate progressively from origin to terminus. Furthermore the speed of the progression of chromosome segregation seems to be linear in time and at a pace similar to that of the replication. This is true for cells growing in glycerol as well as glucose thus covering a growth rate interval from  $I=C+D$  to  $I=C$ . It will be interesting to see if this is true at the fastest growth rates too.

As stated in the accompanying paper (II) about progressive chromosome segregation a progressive segregation at a rate comparable to the rate of replication but delayed with respect to it means that segregation and replication are processes separated in time. It is thus likely that these are entirely independent processes and not related as proposed by the central factory models. Replication leaves the duplicated DNA duplexes linked together somehow and they stay linked together until the segregation process separates them. The SeqA protein is a possible candidate for linking together the newly replicated DNA. The period of sister loci cohesion is important for the cell as known and well described repair systems relying on homologous recombination (repairing of double strand breaks for example) need both daughter duplexes to do the repair. We have shown that segregation of



loci is very efficient; separated loci do not cross back across the cell center (Paper II). Hence the period of sister loci cohesion is probably the only time window where homologous recombination between daughter strands can take place. Whether this is the actual function of the delay period between replication and segregation is not known at present.

### **3.1.2 Separate chromosome arms**

The discovery of a progressive segregation and the determination of its timing with respect to replication is a major breakthrough in the field of chromosome dynamics. With the temporal relationship established we only need to establish the spatial relationship to have a full description of the process of chromosome segregation. This is slightly more difficult to do though as it requires knowledge about the position of replication as well as the position of all chromosomal loci, preferably both before and after segregation.

We do not know the exact position of replication in the cell and it has not been studied in this thesis. What we do know is that there is evidence that markers stay where they were after replication. That is implied from the observations that the single foci in seen in cells that has not replicated and the single foci seen in cells that have (but have not segregated) are apparently positioned similarly in the cell (Paper II). This led us to conclude that markers stay where they are replicated until segregation takes place. However since the intermediate markers then seem to replicate at a non-central position and we would expect replication to be symmetrical around the cell center (that is either a central factory or separated forks on each side) we investigated if intermediate markers from opposite chromosomal arms are positioned in separate cell halves. As reported in Paper III this is indeed the case. Extensive analysis show that this arrangement apparently spans all the way from origin to terminus. This striking and very surprising discovery was also made by Wang and coworkers almost at the same time as we did (Wang *et al.*, 2006).

This result supports, or rather explains, the observation of separating replication forks in slow growing cells (Bates and Kleckner, 2005; Yamazoe *et al.*, 2005). A central factory can be proposed for this chromosomal organization, pulling the DNA in from both sides. Wang *et al.* (2006) do in fact propose such a model. However we disagree as it does not fit with our observations of sister loci cohesion. Newly replicated loci cohere for a short period and our results suggest that this cohesion takes place off-center for the intermediate loci. Thus it is more likely that the intermediate markers are replicated off-center which would result in separating replication forks as observed.

It should be noted that separating forks has not been reported at higher or even moderate growth rates. In fact one study suggests the opposite; that several forks go

together in super-factories at faster growth rates (Molina and Skarstad, 2004). The separation of the two chromosome arms to separate cell halves has also only been observed at slow growth rate. In fact it seems that this organization is not present at higher growth rates (personal observations, not shown). Hence it is possible that replication and segregation follows very different schemes at different growth rates.

### 3.1.3 A model for chromosome segregation

In general the Capture-Extrusion model has received more support (Gordon *et al.*, 2002; Koppes *et al.*, 1999; Lau *et al.*, 2003; Niki *et al.*, 2000; Niki and Hiraga, 1998; Onogi *et al.*, 1999; Roos *et al.*, 2001; Roos *et al.*, 2001) than the Sister Chromosome Cohesion model (Bates and Kleckner, 2005; Hiraga *et al.*, 2000; Sunako *et al.*, 2001). However most of these studies have not compared the two models directly. Often one is under the impression that presented data might as well be interpreted in favor of the competing model. Indeed the data from Hiras lab used to support the Sister Chromosome Cohesion model has been shown to support the Extrusion-Capture Model equally well if reinterpreted (Roos *et al.*, 2001).

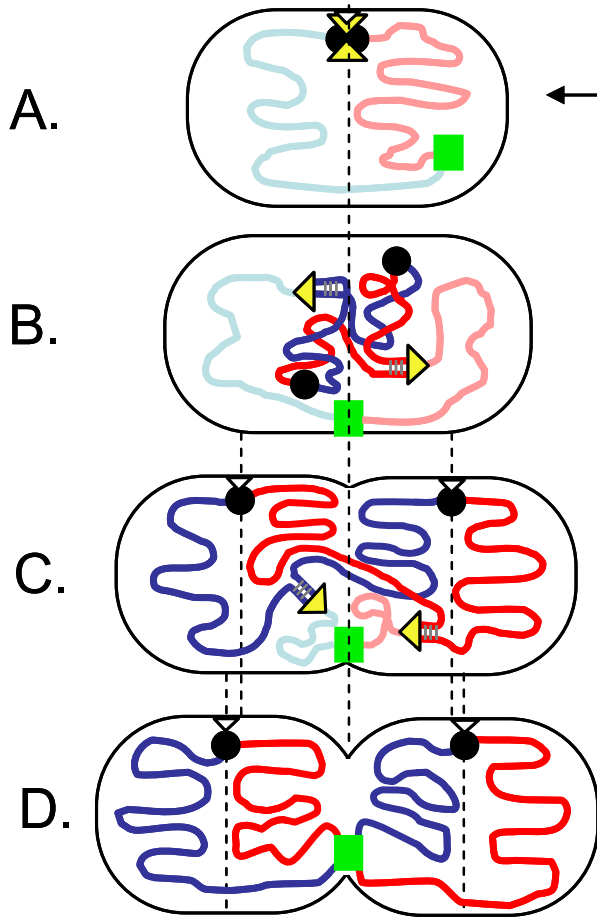
Our finding that the chromosome is organized with its two chromosome arms in separate cell halves is by itself compatible with both models. The recent discoveries that the replication forks split up and migrate to separate cell halves however are not (Bates and Kleckner, 2005; Yamazoe *et al.*, 2005). Of these two models only the Sister Chromosome Cohesion Model is compatible with separating forks.

Our result that the chromosome is progressively segregated on the other hand is clearly not compatible with the Sister Loci Cohesion Model, and because of the observed period of sister loci cohesion it is not compatible with the Extrusion-Capture Model either in its present form. Therefore we have developed a new model that is in accordance with these latest results. This model can be separated into a Sister Loci Cohesion Model and a Home and Away Segregation model as described in section 1.3.

A figure illustrating our current view on chromosome segregation has been presented in Paper III and is reprinted here for convenience as Figure 3.1.

It is important to emphasize that although the Extrusion-Capture Model and the Sister Chromosome Cohesion Model are now discarded because of the results hereby presented both models were quite close to the truth. The progressive segregation predicted by the Extrusion-Capture Model was indeed correct, and only by having precise information about the timing of replication and segregation is it possible to show that these processes are not coincident. Furthermore one should be very careful to discard the idea of a central

replication factory. There is no central factory at the slow growth rates that we and others have used (Wang et al., Bates and Kleckner, Yamazoe et al.) but there is no evidence that there isn't at higher growth rates.



**Figure 3.1 Chromosome rearrangement model.**

A. At initiation, the origin region of the chromosome (black circles) is at the cell center, and the replication forks (yellow triangles) form there. The two arms (light blue and pink lines) of the chromosome are arranged either side of the cell center. The terminus (green square) is near the new cell pole.

B. At mid-replication, the forks have dissociated from the cell center and are following the path of the DNA template. The newly replicated DNA products (dark blue and red) stay together for a while (sister loci cohesion, grey hatching) and then segregate to opposite sides of the cell center. This DNA is relatively disorganized and resides in the central half of the cell.

C. As replication progresses, the new origins attach to the cell quarter positions and the two arms of each nascent chromosome are sorted out so that the arms lie on either side of the cell quarters.

D. At termination, the origins are at the quarter positions, and the terminus and forks are at the cell center. The arms (replichores) of the two new chromosomes are arranged with respect to the cell quarters. After the following post-replicative period (D-period), cell division will restore the starting state of the cycle.

Though the extensive cohesion stated by the Sister Chromosome Cohesion Model is incorrect it is easy to see why it has been proposed. The sister loci cohesion period that we found is quite long (20% of the cell cycle), and thus without a precise and detailed analysis of the chromosome segregation it is easy to misinterpret data as extensive cohesion, as for example it was the case when Bates and Kleckner looked at only 4 markers (Bates and Kleckner, 2005).

Our results thus show that both models are partially correct and explain why evidence supporting both has been available. The chromosomes are progressively segregated but they do also cohere albeit in another way than previously suggested.

### 3.1.4 Capture of the origin

A central part of the Extrusion-Capture model is the directed extrusion of origins towards the cell quarters away from the cell center. In the Sister Chromosome Cohesion model too movement of the origins, as well as most of the chromosome, is also a central part. The first suggests that replication itself provides the force necessary for origin migration whereas the latter suggests a segregation mechanism involving so far unknown molecular motors. The model we have proposed also requires the existence of an unknown active segregation mechanism.

Hence as proposed in all models on chromosome segregation it is expected that origin migration is controlled by the cell. There has however not been found any evidence of this whatsoever. An alternative suggestion has been proposed. It has been observed that the duplicated origins and possibly later replicated markers keep a constant distance to the cell poles moving apart gradually in line with cell elongation (Roos *et al.*, 1999; Roos *et al.*, 2001). This led to the idea that replicated markers segregate passively in line with the axial cell growth. There really are not any reports disputing this idea. However, it would require that growth takes place at the center of the cell. This has been shown not to be true; in fact the cell grows evenly along the entire length of the cell (Cooper, 1991). Thus markers keeping a constant distance to the pole are in fact migrating in the cell as their relative distance from the pole is decreasing. In *Caulobacter crescentus* there is little doubt that origins migrate. They do so very rapidly after duplication (Viollier *et al.*, 2004). One stays at the stalked pole and the other migrates to the opposite pole.

We further believe that all loci are actively segregated by an energy driven mechanism. That is a natural consequence of the Sister Loci Cohesion model and the Home and Away Segregation model. When sister loci cohesion is lost the two sister loci are located outside the cell center on the same side of the middle. They can't stay like that for very long as cells with two foci on the same side of the cell center is a rarity. Instead one seems to jump rapidly across the cell center line and the other stays where it was (the home locus). It is possible that the segregation machinery is located centrally at the future division plane, pulling in the two Away DNA strands from either side of the center and placing the DNA just on the other side of the center. A central segregation machinery would explain why a replication block results in the blocked locus being centrally located along with the replication proteins. That is because the segregation machinery is pulling in the duplicated DNA as soon as the sister loci cohesion is lost. If the replication forks stall they will consequently be pulled to the center where they will jam the segregation machinery. A central segregation apparatus also fits very well with the reports on sticky P1-*parS* loci getting stuck in the center of the cell.

Although the Extrusion-Capture model is wrong about the central replication and extrusion of replicated DNA it might still be right about the origin capture. Origin capture is indeed compatible with the Home and Away Segregation model. In fact capture of the origin could possibly be a central part of the sorting out which chromosomal arm goes to which side. Starting at the origin at a fixed position (the quarter) the chromosome is sorted proceeding towards the terminus putting one arm on one side and the other on the other side.

Evidence of origin capture has been presented in Paper II. We found that addition of rifampicin (blocking initiation) had a great impact on the general distribution of the origins in smaller cells with new origins but very little effect on larger cells with older origins. In the earlier the distribution of origins became very broad whereas the later had the origins narrowly distributed around the cell quarter. This result indicates that the origins are being held at the quarter prior to initiation.

### **3.1.5 General organization of the chromosome**

The chromosome is without doubt organized with the origin at the cell center at cell birth or at least shortly thereafter. The terminus too is at the middle but does not get there until usually a little later in the cell cycle. This depends on the growth rate though; at very slow growth the terminus stays at the new pole for a longer time than at faster growth rates. This feature is by the way useful for identifying the new pole in newborn cells. At cell division when the replication is done and the two chromosomes segregated the conformation is almost the same: The two origins are at the cell quarters (the future cell center). The termini are not at the cell quarters yet, but stays at the division plane; even some time after cell division is complete.

The rest of the chromosome is apparently not very organized at the slow growth rate, except for the fact that one arm or replicore is in one cell half and the other chromosome arm is in the other. Data suggests that these arms are organized with origin proximal markers close to the cell center and later replicated markers closer to the pole when approaching the terminus. At some point around 10' from the terminus this order seems to reverse and markers are again closer to the cell center. This has led to the proposal that the chromosome is organized in a condensed ring structure (Niki *et al.*, 2000). As the distribution of loci in slow growing cells is very broad we don't think this is correct. Instead we propose that the difference in the average positions of the markers reflects the order they were replicated and put into the growing nucleoid mass at the previous segregation event (Nielsen *et al.*, 2006a). In any individual cell an early marker can easily be closer to the pole than a late marker, but on the average they tend to be closer to the cell

center because the early markers were put into the growing nucleoid first and the highly viscous nature of the nucleoid tend to maintain this organization.

The replication forks separate at the low growth rate. As the chromosome arms are in separate cell halves the forks go to each side of the center and probably track along the DNA. We do not know why the forks do not stay together at the cell center pulling the chromosome arms in from either side. Perhaps the general idea of replication factories is incorrect although that does not seem to be true. Separating replication forks have not been reported at higher or even moderate growth rates. It seems intuitively wrong that forks should separate at one growth rate and not at another. If the mechanism of replication does not involve replication factories one would expect to find separating replication forks at any growth rate. Hence at present there is a discrepancy between the observations in cells growing at different growth rates. This discrepancy seems to include the separation of chromosomal arms. Although preliminary, our present data from cells growing in glucose does not support a similar separation of the arms. Perhaps the compactness of the DNA forces the arms to be separated on the short axis of the cell. Replication forks too could be separated on the short axis. It is too early to say at present though.

This is more or less what we know at present about the organization of chromosomal markers and any further details can not come from anything else than qualified guessing. In the literature there only exists extensive and valid data from slowly or very slowly growing cells. The next step is therefore to do extensive analysis as the one reported here (Papers II and III) on cells growing at faster growth rates (at  $I < C$ ).

## 3.2 Perspectives

A lot of questions about bacterial chromosome dynamics have been answered by the results presented here. We are however still at a very early stage and there is still a lot to be learned. Some of the results presented and methods developed in this thesis leads to new questions and to new ideas and approaches in the field. In the following I present these ideas.

### 3.2.1 Implications of the newly developed pMT1 based labeling system

The pMT1 based labeling system was not developed until very recently and has already proven very useful. Naturally the results presented in Paper III about the organization of the chromosomal arms could not have been produced without it. But other experiments are already being planned. With the possibility of labeling two loci independently we can now follow the position of one locus with respect to another. This can be used for inspecting the

position of the two arms with respect to each other as done in Paper III or one locus could be a labeled reference locus that other loci are compared to; for example the terminus that is always close to the new pole. This way the orientation of different cells labeled at different sites can be established with respect to the new pole and a truly oriented distribution of loci in the cell established. Another reference locus could be an intermediate marker representing one of the chromosome arms. In stead of orienting with respect to the new pole cells can then be oriented with respect to the orientation of the chromosome. In both cases the use of a reference locus makes it possible to compare the position of many loci from many different strains without the problem of cell orientation.

An unexpected feature of the pMT1 system was the lack of stickiness. This might turn out to be very useful in segregation studies as explained in the next section, but initially it means that the pMT1 labeling system should be the first choice when doing single color labeling. That is of course because it is not necessary to be so careful when inducing the GFP-ParB. This system can be expressed at high levels without problems. But not only is it an advantage that foci do not stick together but the achievable high levels of GFP minimize problems like bleaching foci and low signal to noise ratios. This will have an even greater impact on time-lapse studies where the bleaching is an especially big problem as the cells are exposed over and over again. With high levels of non-sticky GFP-pMT1-ParB this problem is reduced.

### **3.2.2 Stickiness**

The discovery of stickiness is of great importance and the progressive process of segregation (Paper II) could not have been recognized using the old induction strategy (Li *et al.*, 2002). The protocol we use now for labeling studies is superior to the old method and produces results with no known artifacts as evident from the studies on separation of labeled origins (Paper II). Although that is great for future studies it could force us to reevaluate some of the older results created by using the P1 *par* labeling system. These earlier results mainly involve P1 plasmid partitioning studies published before I began my Ph.D. study. Plasmid partitioning is not a subject of this thesis and I will not begin to discuss the validity of the result I am referring to here, but just mention that it might be advisable to redo some of the experiments.

Two publications where the system has been used to label chromosomal loci using the old induction strategy have been published. In one only the origin and terminus was labeled (Li *et al.*, 2002). Fortunately the localization of these two markers is not greatly affected by stickiness as they tend to localize to the quarters (origin) and the cell center (terminus and origin) anyway which is where they will get stuck by stickiness as well.

Hence only the effect of delay of segregation is a problem. The authors did observe and report this delay. Ironically there really is a delay (sister loci cohesion) although it is not as long as the one reported and caused by stickiness. So overall the paper is still valid even though stickiness was present in the results. The other paper only considered terminal markers (Li *et al.*, 2003). This paper was quite lucky too as the terminus is the locus least affected by stickiness. The terminus localizes to the cell center under normal as well as sticky conditions. The segregation of the terminus is affected though and a lot less cells with two terminus foci observed under sticky conditions, but as the paper was about terminus localization the presence of stickiness had a limited effect on the validity of the reported results. The experiments have been redone recently under conditions without stickiness (Stuart Austin, personal communication, data not shown) and the only difference is that termini separate earlier than originally reported but localize the same way.

Stickiness is likely to be an important feature of the P1 plasmid partitioning process as well as other similar partitioning systems. Indeed the present models for the P1 partitioning mechanism state that two plasmids are held together by ParB proteins at the cell center and then ‘ejected’ very late in the cell cycle by a ParA dependent process (Ebersbach and Gerdes, 2005). This pairing of sister plasmids at the cell center is in line with our observations that pairs of chromosomal *parS* loci are located at the cell center when stickiness is induced. On the other hand the pairing of P1 plasmids at the cell center proposed by the partitioning models could arguably be a result of studying the system at higher than normal levels of ParB where stickiness affects the true partitioning process. If this is the case, stickiness might actually be a problem for the P1 partitioning process that is not overcome until the last minute before cell division. In fact it is not at all impossible that the stuck plasmids delay cell division until they have been segregated properly. The partitioning process of P1 is very efficient and cells practically never divide without a P1 plasmid in each daughter cell, so it would make sense if the plasmid possesses the ability of delaying cell division until segregation is complete. If so that would be a so far unknown feature of the P1 partitioning system. Studies of stickiness’ role in P1 partitioning should therefore be commenced and it should be clarified whether this phenomenon is a part of the partitioning process or not. Existing models should be revalidated to account for the results of such studies. A fact that might speak for the idea of stickiness impeding plasmid partitioning and not being a part of it is that the pMT1 partitioning system does not cause stickiness but still segregates the pMT1 plasmid very efficiently.

Regardless of why stickiness is present in the P1 system and how it works it should prove very useful in chromosome segregation studies. A P1 *parS* locus can be inserted at



one position and segregation blocked at this position by inducing stickiness. The fate of other chromosomal loci can then be followed by a pMT1 *parS* insertion and the following questions answered: Are loci positioned in front of the block (upstream) segregated normally? Are loci after the block segregated? We know that segregation is a progressive process but is that because the segregation tracks along the DNA from origin to terminus, in which case loci in front of the block but not loci after will be expected to segregate normally. Or is the progressive segregation we observe a result of the progressive replication, and the chromosomes segregated progressively simply because the substrate for the segregation machinery is made available in a progressive manner. In the later case both loci before and after the block would be expected to segregate regardless of the segregation block. Finally with the new knowledge that the chromosomal arms are located and replicated separately it would be interesting to check if a segregation block on one arm affects the segregation of the other. Strains suitable for the experiments described here are being made at the moment.

### **3.2.3 Cell counting**

The development of a fully automated cell counting and measuring tool is a major contribution to the field of bacterial chromosome dynamics. How does an *hns* mutation affect the general chromosome segregation? Or what about *fis*, *fis*, or *dnaA* mutations to mention a few other interesting candidates. Is the general chromosome organization affected? To answer these questions the segregation and localization pattern of many chromosomal markers in strains with the mentioned mutations has to be determined easily involving the measuring of more than 10.000 cells for each mutant. That would be close to impossible to do or at least extremely time consuming without an automated procedure. With the program described in Paper I this is now possible. Experiments that would normally take weeks to do or perhaps never even be commenced without the program can now be done in fractions of the time.

The main results of this thesis are without doubt the discovery that chromosome segregation is progressive and that the chromosomal arms are located in separate cell halves of slow growing cells as reported in Paper II and III respectively. It had however not been possible to get these results without the automated cell counting and measuring program. This program is therefore also a very important result of my work and will hopefully contribute to many future studies by our laboratory and others that would like to use it.

### 3.2.4 Future of still image analysis

Many people criticize the use of still image analysis for determining the dynamics of chromosomal loci as this kind of analysis does not include the time dimension. I think it is fair to say that this thesis has shown that still image analysis can be extremely valuable. First of all it is not only the movement of loci that is under investigation but also the general localization pattern (which one can argue are two sides of the same story). The general position of a locus in the cell as well as its scatter tells a lot about the dynamics of this locus. The time dimension we get from the cell size as this is a time dependent variable. Furthermore we have shown that time-lapse studies are very difficult to rely on as loci are so mobile that a record of their position every 15<sup>th</sup>, 10<sup>th</sup>, or even 5<sup>th</sup> minute does not say much as there is no information on their positions in between. It becomes a series of still images of the same cell that is difficult to connect in time, and hence does not differ much from a normal still image analysis except that a lot fewer cells are analysed. This being said time-lapse and still image analysis can of course be used to confirm one another.

I believe still image analysis have a great future in chromosome dynamics. With the development of an automated counting and measuring method many cells from many different growth conditions and with various strain backgrounds can be analysed in no time. Time-lapse studies which are much more time consuming can then be done on promising strains as a verification method if desired. The statistical basis made from analyzing 2,000 cells is very good and can possibly reveal details that time-lapse would never have had. If 2,000 cells isn't enough we just analyse 10,000.

With the use of several labeled loci at the same time possibly in combination with GFP fused proteins like SeqA, DnaX, FtsZ etc. still image analysis becomes even more promising as there is really no practical limit to the number of combinations that can possibly be analysed by this technique.

## 4 Bibliography

Adachi, S., Hori, K., and Hiraga, S. (2006) Subcellular positioning of F plasmid mediated by dynamic localization of SopA and SopB. *J Mol Biol* **356**: 850-863.

Adachi, S., Kohiyama, M., Onogi, T., and Hiraga, S. (2005) Localization of replication forks in wild-type and mukB mutant cells of Escherichia coli. *Mol Genet Genomics* **274**: 264-271.

Atlung, T., and Hansen, F.G. (1993) Three distinct chromosome replication states are induced by increasing concentrations of DnaA protein in Escherichia coli. *J Bacteriol* **175**: 6537-6545.

Bakker, A., and Smith, D.W. (1989) Methylation of GATC sites is required for precise timing between rounds of DNA replication in Escherichia coli. *J Bacteriol* **171**: 5738-5742.

Bates, D., and Kleckner, N. (2005) Chromosome and replisome dynamics in E. coli: loss of sister cohesion triggers global chromosome movement and mediates chromosome segregation. *Cell* **121**: 899-911.

Begg, K.J., and Donachie, W.D. (1978) Changes in cell size and shape in thymine-requiring Escherichia coli associated with growth in low concentrations of thymine. *J Bacteriol* **133**: 452-458.

Boye, E., and Lobner-Olesen, A. (1990) The role of dam methyltransferase in the control of DNA replication in E. coli. *Cell* **62**: 981-989.

Boye, E., and Lobner-Olesen, A. (1991) Bacterial growth control studied by flow cytometry. *Res Microbiol* **142**: 131-135.

Boye, E., Steen, H.B., and Skarstad, K. (1983) Flow cytometry of bacteria: a promising tool in experimental and clinical microbiology. *J Gen Microbiol* **129**: 973-980.

Boye, E., Stokke, T., Kleckner, N., and Skarstad, K. (1996) Coordinating DNA replication initiation with cell growth: differential roles for DnaA and SeqA proteins. *Proc Natl Acad Sci U S A* **93**: 12206-12211.

Brendler, T., and Austin, S. (1999) Binding of SeqA protein to DNA requires interaction between two or more complexes bound to separate hemimethylated GATC sequences. *EMBO J* **18**: 2304-2310.

- Brendler, T., Sawitzke, J., Sergueev, K., and Austin, S. (2000) A case for sliding SeqA tracts at anchored replication forks during *Escherichia coli* chromosome replication and segregation. *EMBO J* **19**: 6249-6258.
- Churchward, G., Estiva, E., and Bremer, H. (1981) Growth rate-dependent control of chromosome replication initiation in *Escherichia coli*. *J Bacteriol* **145**: 1232-1238.
- Cooper, S. (1991) Synthesis of the cell surface during the division cycle of rod-shaped, gram-negative bacteria. *Microbiol Rev* **55**: 649-674.
- Cooper, S., and Helmstetter, C.E. (1968) Chromosome replication and the division cycle of *Escherichia coli* B/r. *J Mol Biol* **31**: 519-540.
- Deibler, R.W., Rahmati, S., and Zechiedrich, E.L. (2001) Topoisomerase IV, alone, unknots DNA in *E. coli*. *Genes Dev* **15**: 748-761.
- Dingman, C.W. (1974) Bidirectional chromosome replication: some topological considerations. *J Theor Biol* **43**: 187-195.
- Donachie, W.D. (1968) Relationship between cell size and time of initiation of DNA replication. *Nature* **219**: 1077-1079.
- Ebersbach, G., and Gerdes, K. (2005) Plasmid segregation mechanisms. *Annu Rev Genet* **39**: 453-479.
- Fekete, R.A., and Chattoraj, D.K. (2005) A cis-acting sequence involved in chromosome segregation in *Escherichia coli*. *Mol Microbiol* **55**: 175-183.
- Fujikawa, N., Kurumizaka, H., Nureki, O., Tanaka, Y., Yamazoe, M., Hiraga, S., and Yokoyama, S. (2004) Structural and biochemical analyses of hemimethylated DNA binding by the SeqA protein. *Nucleic Acids Res* **32**: 82-92.
- Gordon, G.S., Shivers, R.P., and Wright, A. (2002) Polar localization of the *Escherichia coli* oriC region is independent of the site of replication initiation. *Mol Microbiol* **44**: 501-507.
- Gordon, G.S., Sitnikov, D., Webb, C.D., Teleman, A., Straight, A., Losick, R. *et al.* (1997) Chromosome and low copy plasmid segregation in *E. coli*: visual evidence for distinct mechanisms. *Cell* **90**: 1113-1121.
- Guarne, A., Brendler, T., Zhao, Q., Ghirlando, R., Austin, S., and Yang, W. (2005) Crystal structure of a SeqA-N filament: implications for DNA replication and chromosome organization. *EMBO J* **24**: 1502-1511.
- Guarne, A., Zhao, Q., Ghirlando, R., and Yang, W. (2002) Insights into negative modulation of *E. coli* replication initiation from the structure of SeqA-hemimethylated DNA complex. *Nat Struct Biol* **9**: 839-843.

Hansen, F.G., Christensen, B.B., and Atlung, T. (1991) The initiator titration model: computer simulation of chromosome and minichromosome control. *Res Microbiol* **142**: 161-167.

Helmstetter, C.E. (1996) Timing of synthetic activities in the cell cycle. In *Escherichia coli and Salmonella cellular and molecular biology*. Neidhardt, F.C., J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter, and H.E. Umbarger, eds. Washington D.C: ASM Press, pp. 1627-1639.

Helmstetter, C.E., and Cummings, D.J. (1963) Bacterial Synchronization by selection of cells at division. *Proc Natl Acad Sci U S A* **50**: 767-774.

Helmstetter, C.E., Eenhuis, C., Theisen, P., Grimwade, J., and Leonard, A.C. (1992) Improved bacterial baby machine: application to *Escherichia coli* K-12. *J Bacteriol* **174**: 3445-3449.

Helmstetter, C.E., and Pierucci, O. (1976) DNA synthesis during the division cycle of three substrains of *Escherichia coli* B/r. *J Mol Biol* **102**: 477-486.

Hiraga, S., Ichinose, C., Niki, H., and Yamazoe, M. (1998) Cell cycle-dependent duplication and bidirectional migration of SeqA-associated DNA-protein complexes in *E. coli*. *Mol Cell* **1**: 381-387.

Hiraga, S., Ichinose, C., Onogi, T., Niki, H., and Yamazoe, M. (2000) Bidirectional migration of SeqA-bound hemimethylated DNA clusters and pairing of *oriC* copies in *Escherichia coli*. *Genes Cells* **5**: 327-341.

Jensen, R.B., and Shapiro, L. (1999) The *Caulobacter crescentus* *smc* gene is required for cell cycle progression and chromosome segregation. *Proc Natl Acad Sci U S A* **96**: 10661-10666.

Koch, A.L. (1996) Similarities and Differences of Individual Bacteria within a Clone. In *Escherichia coli and Salmonella cellular and molecular biology*. Neidhardt, F.C., J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter, and H.E. Umbarger, eds. Washington D.C: ASM Press, pp. 1640-1651.

Koppes, L.J., and Nanninga, N. (1980) Positive correlation between size at initiation of chromosome replication in *Escherichia coli* and size at initiation of cell constriction. *J Bacteriol* **143**: 89-99.

Koppes, L.J., Woldringh, C.L., and Nanninga, N. (1999) *Escherichia coli* contains a DNA replication compartment in the cell center. *Biochimie* **81**: 803-810.

Lau, I.F., Filipe, S.R., Soballe, B., Okstad, O.A., Barre, F.X., and Sherratt, D.J. (2003) Spatial and temporal organization of replicating *Escherichia coli* chromosomes. *Mol Microbiol* **49**: 731-743.

Lemon, K.P., and Grossman, A.D. (1998) Localization of bacterial DNA polymerase: evidence for a factory model of replication. *Science* **282**: 1516-1519.

Lemon, K.P., and Grossman, A.D. (2000) Movement of replicating DNA through a stationary replisome. *Mol Cell* **6**: 1321-1330.

Lemon, K.P., and Grossman, A.D. (2001) The extrusion-capture model for chromosome partitioning in bacteria. *Genes Dev* **15**: 2031-2041.

Li, Y., Sergueev, K., and Austin, S. (2002) The segregation of the Escherichia coli origin and terminus of replication. *Mol Microbiol* **46**: 985-996.

Li, Y., Youngren, B., Sergueev, K., and Austin, S. (2003) Segregation of the Escherichia coli chromosome terminus. *Mol Microbiol* **50**: 825-834.

Massey, T.H., Aussel, L., Barre, F.X., and Sherratt, D.J. (2004) Asymmetric activation of Xer site-specific recombination by FtsK. *EMBO Rep* **5**: 399-404.

Messer, W.J., and Weigel, C. (1996) Initiation of Chromosome Replication. In Escherichia coli and Salmonella cellular and molecular biology. Neidhardt, F.C., J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter, and H.E. Umbarger, eds. Washington D.C: ASM Press, pp. 1579-1601.

Michelsen, O., Teixeira de Mattos, M.J., Jensen, P.R., and Hansen, F.G. (2003) Precise determinations of C and D periods by flow cytometry in Escherichia coli K-12 and B/r. *Microbiology* **149**: 1001-1010.

Molina, F., and Skarstad, K. (2004) Replication fork and SeqA focus distributions in Escherichia coli suggest a replication hyperstructure dependent on nucleotide metabolism. *Mol Microbiol* **52**: 1597-1612.

Nielsen, H.J., Li, Y., Youngren, B., Hansen, F.G., and Austin, S. (2006a) Progressive segregation of the Escherichia coli chromosome. *Mol Microbiol* **61**: 383-393.

Nielsen, H.J., Ottesen, J.R., Youngren, B., Hansen, F.G., and Austin, S. (2006b) The Escherichia coli chromosome is organized with the left and right chromosome arms in separate cell halves. *Mol Microbiol* **62**: 331-338.

Niki, H., and Hiraga, S. (1998) Polar localization of the replication origin and terminus in Escherichia coli nucleoids during chromosome partitioning. *Genes Dev* **12**: 1036-1045.

Niki, H., and Hiraga, S. (1999) Subcellular localization of plasmids containing the oriC region of the Escherichia coli chromosome, with or without the sopABC partitioning system. *Mol Microbiol* **34**: 498-503.

Niki, H., Yamaichi, Y., and Hiraga, S. (2000) Dynamic organization of chromosomal DNA in Escherichia coli. *Genes Dev* **14**: 212-223.

- Okazaki, T., and Okazaki, R. (1969) Mechanism of DNA chain growth. IV. Direction of synthesis of T4 short DNA chains as revealed by exonucleolytic degradation. *Proc Natl Acad Sci U S A* **64**: 1242-1248.
- Onogi, T., Niki, H., Yamazoe, M., and Hiraga, S. (1999) The assembly and migration of SeqA-Gfp fusion in living cells of *Escherichia coli*. *Mol Microbiol* **31**: 1775-1782.
- Pelletier, A.J., Hill, T.M., and Kuempel, P.L. (1988) Location of sites that inhibit progression of replication forks in the terminus region of *Escherichia coli*. *J Bacteriol* **170**: 4293-4298.
- Pierucci, O., and Helmstetter, C.E. (1976) Chromosome segregation in *Escherichia coli* B/r at various growth rates. *J Bacteriol* **128**: 708-716.
- Possoz, C., Filipe, S.R., Grainge, I., and Sherratt, D.J. (2006) Tracking of controlled *Escherichia coli* replication fork stalling and restart at repressor-bound DNA in vivo. *EMBO J* **25**: 2596-2604.
- Roos, M., Lingeman, R., Woldringh, C.L., and Nanninga, N. (2001) Experiments on movement of DNA regions in *Escherichia coli* evaluated by computer simulation. *Biochimie* **83**: 67-74.
- Roos, M., van Geel, A.B., Aarsman, M.E., Veuskens, J.T., Woldringh, C.L., and Nanninga, N. (1999) Cellular localization of *oriC* during the cell cycle of *Escherichia coli* as analyzed by fluorescent in situ hybridization. *Biochimie* **81**: 797-802.
- Roos, M., van Geel, A.B., Aarsman, M.E., Veuskens, J.T., Woldringh, C.L., and Nanninga, N. (2001) The replicated *ftsQAZ* and *minB* chromosomal regions of *Escherichia coli* segregate on average in line with nucleoid movement. *Mol Microbiol* **39**: 633-640.
- Sawitzke, J., and Austin, S. (2001) An analysis of the factory model for chromosome replication and segregation in bacteria. *Mol Microbiol* **40**: 786-794.
- Sherratt, D.J., Soballe, B., Barre, F.X., Filipe, S., Lau, I., Massey, T., and Yates, J. (2004) Recombination and chromosome segregation. *Philos Trans R Soc Lond B Biol Sci* **359**: 61-69.
- Skarstad, K., Boye, E., and Steen, H.B. (1986) Timing of initiation of chromosome replication in individual *Escherichia coli* cells. *EMBO J* **5**: 1711-1717.
- Skarstad, K., Steen, H.B., and Boye, E. (1985) *Escherichia coli* DNA distributions measured by flow cytometry and compared with theoretical computer simulations. *J Bacteriol* **163**: 661-668.

Slater, S., Wold, S., Lu, M., Boye, E., Skarstad, K., and Kleckner, N. (1995) E. coli SeqA protein binds oriC in two different methyl-modulated reactions appropriate to its roles in DNA replication initiation and origin sequestration. *Cell* **82**: 927-936.

Straight, A.F., Belmont, A.S., Robinett, C.C., and Murray, A.W. (1996) GFP tagging of budding yeast chromosomes reveals that protein-protein interactions can mediate sister chromatid cohesion. *Curr Biol* **6**: 1599-1608.

Sunako, Y., Onogi, T., and Hiraga, S. (2001) Sister chromosome cohesion of Escherichia coli. *Mol Microbiol* **42**: 1233-1241.

Sundin, O., and Varshavsky, A. (1981) Arrest of segregation leads to accumulation of highly intertwined catenated dimers: dissection of the final stages of SV40 DNA replication. *Cell* **25**: 659-669.

Viollier, P.H., Thanbichler, M., McGrath, P.T., West, L., Meewan, M., McAdams, H.H., and Shapiro, L. (2004) Rapid and sequential movement of individual chromosomal loci to specific subcellular locations during bacterial DNA replication. *Proc Natl Acad Sci U S A* **101**: 9257-9262.

von Freiesleben, U., Krekling, M.A., Hansen, F.G., and Lobner-Olesen, A. (2000) The eclipse period of Escherichia coli. *EMBO J* **19**: 6240-6248.

von Freiesleben, U., Rasmussen, K.V., and Schaechter, M. (1994) SeqA limits DnaA activity in replication from oriC in Escherichia coli. *Mol Microbiol* **14**: 763-772.

Wang, L., and Lutkenhaus, J. (1998) FtsK is an essential cell division protein that is localized to the septum and induced as part of the SOS response. *Mol Microbiol* **29**: 731-740.

Wang, X., Liu, X., Possoz, C., and Sherratt, D.J. (2006) The two Escherichia coli chromosome arms locate to separate cell halves. *Genes Dev* **20**: 1727-1731.

Wang, X., Possoz, C., and Sherratt, D.J. (2005) Dancing around the divisome: asymmetric chromosome segregation in Escherichia coli. *Genes Dev* **19**: 2367-2377.

Webb, C.D., Teleman, A., Gordon, S., Straight, A., Belmont, A., Lin, D.C. *et al.* (1997) Bipolar localization of the replication origin regions of chromosomes in vegetative and sporulating cells of B. subtilis. *Cell* **88**: 667-674.

Yamaichi, Y., and Niki, H. (2004) migS, a cis-acting site that affects bipolar positioning of oriC on the Escherichia coli chromosome. *EMBO J* **23**: 221-233.

Yamazoe, M., Adachi, S., Kanaya, S., Ohsumi, K., and Hiraga, S. (2005) Sequential binding of SeqA protein to nascent DNA segments at replication forks in synchronized cultures of Escherichia coli. *Mol Microbiol* **55**: 289-298.



Youngren, B., Radnedge, L., Hu, P., Garcia, E., and Austin, S. (2000) A plasmid partition system of the P1-P7par family from the pMT1 virulence plasmid of *Yersinia pestis*. *J Bacteriol* **182**: 3924-3928.



## 5 Co-author statements

### Paper I

Nielsen, Henrik J. & Hansen, Flemming G. (2006). An automated and highly efficient method for counting and measuring foci in rod shaped bacteria. *Manuscript*.

All programming and experiments were carried out by HJN. The manuscript was written by HJN supervised by FGH.



Henrik J Nielsen



Flemming G. Hansen

### Paper II

Nielsen, Henrik J., Li, Yongfang, Youngren, Brenda, Hansen, Flemming G. & Austin, Stuart (2006). Progressive segregation of the *Escherichia coli* chromosome. *Molecular Microbiology* **61**(2), 383-393.

Strain constructions were done by YL. Growth experiments and microscopy were done by BY and HJN. Data analysis was done by HJN. The manuscript was written by HJN supervised by FGH and SJA, and submitted by FGH.



Henrik J Nielsen



Brenda Youngren



Youngfang Li



Stuart J Austin



Flemming G. Hansen

**Paper III**

Nielsen, Henrik J., Ottesen, Jesper R., Youngren, Brenda, Austin, Stuart & Hansen, Flemming G. (2006). The *Escherichia coli* chromosome is organized with each chromosome arm in separate cell halves. *Molecular Microbiology* **62**(2),331-338.

Strain constructions were done by HJN. Plasmid constructions were done by JRO and FGH. Growth experiments and microscopy were done by BY and HJN. Data analysis was done by HJN. The manuscript was written by HJN supervised by FGH and SJA, and submitted by FGH.

  
Henrik J Nielsen

  
Brenda Youngren

  
Jesper R. Ottesen

  
Stuart J. Austin

  
Flemming G. Hansen

## Papers and manuscripts

Paper I	An automated and highly efficient method for counting and measuring foci in rod shaped bacteria	6 pages
Paper II	Progressive segregation of the <i>Escherichia coli</i> chromosome	11 pages
	Supplementary material for Paper II	1 page
Paper III	The <i>Escherichia coli</i> chromosome is organized with the left and right chromosome arms in separate cell halves	8 pages
	Supplementary material for Paper III	3 pages



# An automated and highly efficient method for counting and measuring foci in rod shaped bacteria

Henrik J. Nielsen, Flemming G. Hansen\*

Center for Biological Sequence Analysis, BioCentrum-DTU, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

## Summary

Measurements directly on photo micrographs of cells are becoming increasingly more used when investigating the position and/or distribution of chromosomal loci in bacteria. These measurements have in many cases been done manually, and without clear definitions on how they are measured. Here we present a procedure for standardizing measurements of cell properties on phase contrast images. Furthermore we present a program using these standardized methods capable of measuring the intracellular positions of fluorescent foci in bacterial cells faster and more precise than manual measuring.

## Introduction

Unlike eukaryotes, bacteria do not have their chromosomes contained in a nucleus. Instead the chromosome is placed in the cytosol occupying much of the cell volume. As for all cells, the bacterium replicates the chromosome once per division cycle and segregates the daughter chromosomes to two new daughter cells. Very little is known about this process though. What makes it complicated is that the process appears to be continuous and coincident with replication (Nielsen *et al.*, 2006a; Nielsen *et al.*, 2006b) Furthermore many fast growing bacteria are capable of having multiple rounds of replication taking place simultaneously on the same chromosome. These cells have to segregate replicating and highly branched chromosomes before cell division.

Investigating the chromosome dynamics of bacteria has been practically impossible until the recent development of methods for determining the physical positions of specific proteins and DNA sequences in the bacterial cell. Our understanding of chromosome duplication and segregation dynamics has been greatly

enhanced by the use of these techniques. Loci can be detected by fluorescence in fixed cells by in situ hybridization (Niki and Hiraga, 1998). By using fluorescent techniques such as the GFP-ParB/*parS* system (Li *et al.*, 2002) or the GFP-LacI/operator and Tet repressor/operator systems (Gordon *et al.*, 1997; Lau *et al.*, 2003; Wang *et al.*, 2006), it has become possible to detect the position of discrete DNA loci inside the living cell. Using these techniques, it is possible to determine the movement of individual parts of the chromosome as they are replicated and segregated. Monitoring the number of foci as function of cell length provides information on the timing of segregation of a given locus and measurements of the relative position of the foci inside the cell provides information on the spatial dynamics of segregation of that particular labeled locus. It is of course crucial that the determination of cell size and foci number as well as coordinates is as accurate as possible.

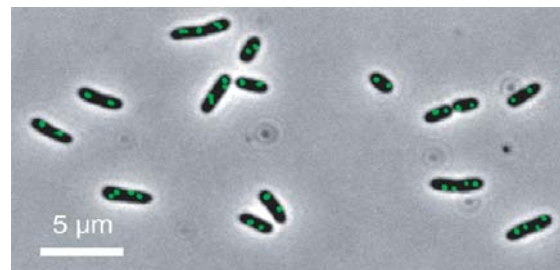


Fig. 1. Phase contrast photomicrograph of *Escherichia coli* K-12 (MG1655) cells.

Measurements are done on photomicrographs of fluorescent foci inside the cells from a fluorescence microscope and photomicrographs of the same cells from phase contrast microscopy. The intracellular number and position of foci can be determined by overlaying the fluorescent image on the phase contrast image and measuring the positions and dimension of cells and foci (Figure 1). This is typically done manually using a digital analysis program and is very time consuming and has some observer bias problems. Furthermore it is our experience that at least 1000 cells are needed for each labeled chromosome locus in order to get significant results. This is due to considerable cell to cell variation in focus number and intracellular position. Manual analysis

\*For correspondence. E-mail fgh@biocentrum.dtu.dk; Tel. (+45) 45 25 25 05; Fax (+45) 45 93 28 09.

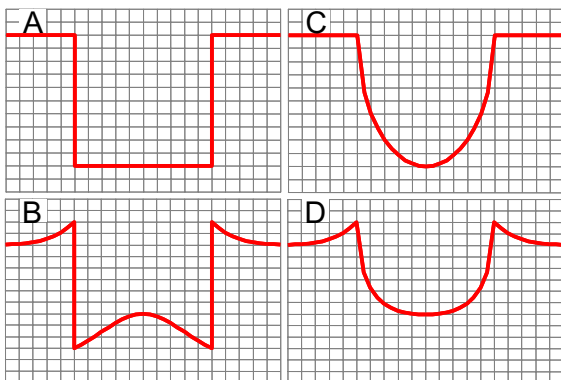
of this number of cells takes at least 4 hours depending on the software used.

We have created/programmed a macro capable of measuring cell properties and foci positions fully automatically and very efficiently. It has no observer bias, it gives less variation in measurements than humanly measured cells, and most importantly it will easily count and measure more than 1000 cells in 5 minutes. The macro was programmed in the digital image processing and analysis program Image Pro Plus.

## Results

### *Determining the true outline of the cells on phase contrast images*

It is well known that the edges of objects and especially cells observed in phase contrast are not sharp. In phase contrast microscopy the refractory difference of the object compared to the surrounding media is visualized as an intensity difference. Depending on the thickness of the object and its refractory index, a phenomenon creating a bright fringe (halo, figure 1) around the object is present to a more or less troubling degree obscuring edge details (Bennett *et al.*, 1951). This is especially unfortunate when doing measurements on cylindrically (rod) shaped cells as the 'true' edge or outline of the cell is difficult to determine. This is shown in figure 2.

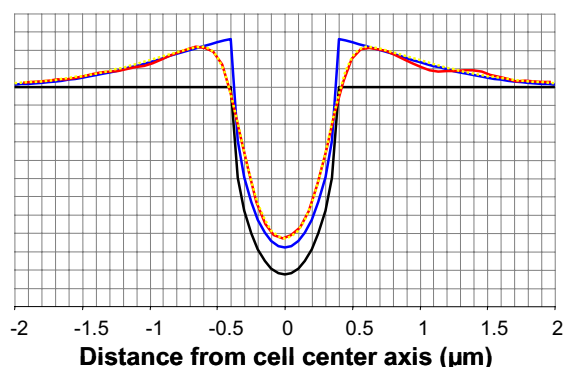


**Fig. 2.** The 'halo' effect. A schematic representation of how the 'halo' effect in phase contrast microscopy changes the true image intensity profile of the specimen (A and C) to a distorted intensity profile (B and D). A and B represent an object with sharp edges like a box and C and D represent a cylindrical object like a rod-shaped cell.

When outlining cells automatically using digital image analysis software it is done by creating a binary mask. This mask is white in all areas where the intensity value of the image is below a certain threshold and black where the intensity of the image is above. When the threshold value is set somewhere between the image

background intensity and the average intensity of the cells the mask contains all the cells, white on black background. The software can then identify and outline these white cells automatically. The actual outlining of the cells depends on the precise value of the threshold. A range of thresholds will usually work fine for creating nice separated outlines, but the size of the final outlined cells will vary considerably depending on the exact threshold value. Hence determining the correct threshold value is important in order to avoid big variations in measured cell size from image to image.

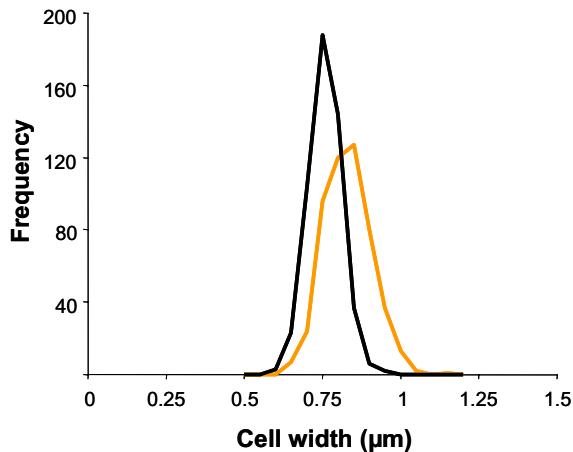
An intensity profile of an *E. coli* cell is shown in figure 3. As can be seen, because of the 'halo' effect, the true outline of rod shaped cells should be placed close to the intensity value of the background. That is however difficult to do in practice because a threshold value close to that of the background level will include a lot of the background. That could be overcome by using a more advanced method for creating the mask, but still it would cause problems with too many cells not being separated. Instead we chose to set the value at 2/3's the range from the cell center intensity and that of the background. That creates outlines that are slightly smaller than the actual cell size but are considerably easier to make. That is not a problem as it is not as important to find the exact true edge of the cell as it is to be consistent from cell to cell when determining the outline.



**Fig. 3.** Example of a cell intensity profile. The short axis intensity profile of an *E. coli* cell growing in AB glycerol minimal media is shown in red. The best guess of the 'true' intensity profile is shown in black. This best guess was found by applying the distortion pattern demonstrated in figure 2 to theoretical cylindrical objects of varying radii and adding a Gaussian distribution to simulate the experimentally observed blurring of the cell. The distorted profile of the black theoretical curve is shown in blue and the blurred version in yellow. The cylindrical radius giving the best fit was then chosen. As seen the yellow curve fits very well with the experimentally determined intensity profile. The threshold value resulting in outlining of the entire cell is right at the background level.

This method does probably not get the entire cell included in the outline but it will outline the cells very consistently so that the measurement error is the same





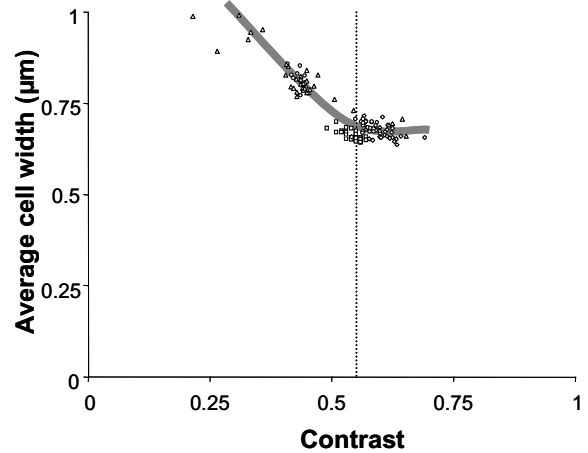
**Fig. 4.** Comparison of manual and automatic measurements. The width of 508 cells were measured manually (yellow curve) and automatically (black curve). The manual measurement resulted in an average cell width of 0.83  $\mu\text{m}$ , a standard deviation of 0.08 and a coefficient of variation of 9.1%. The automatic measurement resulted in an average cell width of 0.76  $\mu\text{m}$ , a standard deviation of 0.05 and a coefficient of variation of 6.9%.

for all cells. Because the error is consistent (estimated to 0.05 microns for all edges) it does not introduce variability to the later measurements on relative position of foci inside the cell. If desired the measurements can be compensated as the magnitude of this error is known. Thus the automatically outlining produces more consistent and more accurate measurements than manual measurements do.

To confirm that this method is consistent we evaluated the variation of the measured width of the cells from image to image compared to the variation from cell to cell. The width of the cells is expected to be more or less the same for all cells and is therefore very useful for determining the robustness of the method.

In figure 4 the result is shown. 507 cells were measured manually and automatically. The average cell width was slightly higher when manually measured. This is of course very dependent on the person doing the measurements. The variation was clearly higher for the manual measurements too (see legend to figure 4 for exact numbers). Hence we conclude that automatic measuring of cells is more consistent than manual measurements. Furthermore it should be noted that the manual measuring of the 500 cells took 30 minutes, and that was without measuring anything else like foci positioning etc. The automatic measuring procedure took 3 minutes, and that was for the entire dataset of 1753 cells and including measurements of cell width, length and foci coordinates.

Once the proper threshold value is found it is no problem to find, outline, and measure all the cells on



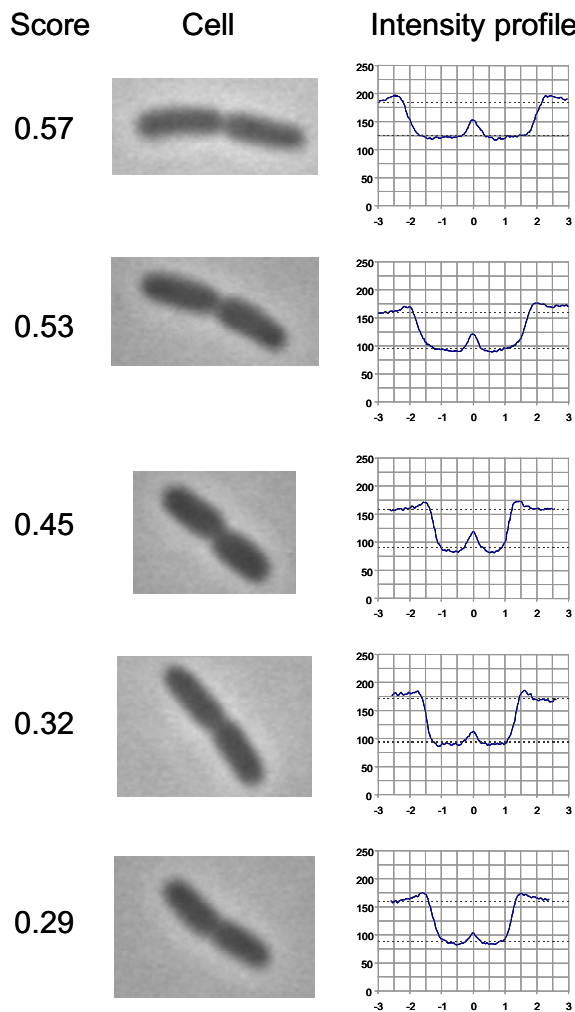
**Fig. 5.** Image contrast and measured cell size. The average cell width was measured using the automated method on images of different cell to background contrast. Each point represents one image from one of four separate experiments (shown by 4 different symbols). Cells were grown similarly in all experiments. At low contrast values the cells appeared wider, but above a contrast value of approximately 0.55 (hatched line) the width of the cells become constant. The trend is shown by the thick grey curve.

each field automatically using the digital image analysis software. Problems with cells lying close together being counted as one, and debris on the image being counted as cells, were easily solved by using measurement filters. Every digital image analysis program has the option of using these filters where all objects above or below a certain length, width, and aspect ratio, are filtered (see Methods for specific values).

Finally we would like to note that only images with cell to background contrast values above 0.55 were used. Images that are slightly out of focus will have a low contrast value. Also dried up cells appears lighter in phase contrast and results in lower contrast values. The limit of 0.55 turned out to be a proper limit for getting rid of out of focus images and images of dried up cells. The variation of cell width was much higher at contrast values below 0.55 but low and constant at contrast values above this limit (figure 5).

#### *Scoring septated and dividing cells*

For many analyses on chromosome dynamics and the bacterial cell division cycle it is of particular interest to isolate the dividing cells and analyze these separately. That is because the cell size is not a precise measure of progression of the cell cycle. The size distribution of cells about to divide is rather broad (CV at 20%; (Koch, 1996). Of course the same is true for newborn cells. Selecting for septated cells is a much better way of isolating cells close to cell division. Furthermore the degree of



**Fig. 6.** Detecting dividing cells. Examples of dividing cells with different invagination scores (left column; scored as described in the text) are shown along with their intensity profiles (right column). The hatched lines represents the background intensity (the upper line) and the cell intensity (the lower line). The blue curve is the intensity profile of the cell along its long axis. The x-axis is the distance from the septum in  $\mu\text{m}$  and the y-axis is the intensity (8 bit).

invagination shows exactly how close the cell is to division. So we decided to have the macro identify septated cells and give them a score depending on the degree of invagination. This was done by creating an intensity profile for each cell. The cell is sliced up along the long axis (as a sausage) and the integrated intensity of each slice calculated. If the cell is invaginated the central slices will be of higher intensity. The degree of invagination is found from the intensity of the brightest point (or 'slice') in the central 1/3 of the cell compared to the cell average intensity and the intensity of the background such that if the brightest point is the same intensity as the rest of the cell the result is zero and if the

brightest point is as bright as the background the score is 1 (see figure 6 for examples). This method was remarkably accurate. In general cells with invagination scores below 0.25 are rarely visibly invaginated, cells with a score between 0.25 and 0.45 are clearly invaginated but not divided, and cells scoring 0.45 or more almost always look like divided cells (see figure 6).

The calculation of the invagination of the cells is done automatically by the macro during the automatic counting and measuring of cells. It is fast and has no observer biased method for detecting septated and dividing cells.

#### *Determining the position of foci automatically*

The next step is to go through each and every cell 'individually' and count the foci within. This was done on the fluorescent images using the data on cell dimensions obtained from the phase contrast images. Basically the problem is the same as for the counting of cells, namely to find the right threshold value. As for the cells this can be done for the entire image, counting all the foci on the image and subsequently assigning them to the previously counted cells. However doing the counting of foci individually for each cell, instead of for the entire image, is much more advantageous, because the fluorescence in some cells are usually much higher than in others.

Individual counting allows us to use a different threshold value for each cell. This threshold value was found as described below.

The threshold value has to be sufficiently above the background intensity level in the cell. If it isn't, background noise can be picked up as signal. A lower limit of the threshold value was therefore set at the value where 85% of the pixels (inside the cell) are darker. This 85% limit was chosen empirically and turned out to be very useful. Likewise an upper limit at 95% was set as foci containing cells with less than 5% of their area consisting of foci are seldom seen. Finally this range of intensity values corresponding to the brightest 85%-95% of the cell area was divided into seven intervals that were evaluated individually for their usefulness as thresholds. The highest threshold value giving the highest number of foci was used. At the same time it is a requirement that foci are equal in size and intensity in order not to pick up noise together with a real signal (see Methods). This procedure was done for each and every cell.

Contrary to the cell outlining, the fidelity of manual identification of foci is generally superior to automatic identification. Foci are sometimes missed when counting them automatically. The reported method however got the number of foci right in 998 out of 1008 cells corresponding to 99% of all cells. Evaluation of the automatic counting was done by comparing the number of foci found by manual counting to the number found by

automatic counting (see table 1). The cells were first counted fully automatically and then every cell was inspected manually. The counting was done in two minutes whereas the visual inspection (without measuring anything) took 30 minutes.

## Discussion

The goal of this work was to develop a fully automatic method for measuring cell dimensions, number of foci, and foci coordinates. This goal was achieved. The method produces consistent results that are without observer bias. More importantly the method is several orders of magnitudes faster than manual counting. That allows the processing of large number of experiments in very little time providing us with new possibilities in the field of bacterial chromosome dynamics. Using this program the effect of many different growth conditions or mutations on the chromosome dynamics can be investigated in no time. The method has already been used in a recently published paper where 14 different strains and a total of more than 16000 cells were analyzed (Nielsen *et al.*, 2006a). This analysis that would have taken more than fifty hours of manual work was done in less than one hour.

Several papers on bacterial chromosome dynamics has been published in the recent years, all using fluorescent labeling systems labeling specific chromosomal loci followed by measurements of foci number and positions. The method and program reported here can help the reporting laboratories and save them a lot of time with these kind of analyses.

Changes can easily be made to the program to make it suitable for other growth conditions or other kinds of bacteria or even for eukaryotic cells. It can also be altered to other tasks like measuring the position of cytoskeletal structures as the FtsZ ring or specific

membrane proteins.

## Material and methods

### Strains and growth media

The strains used were *Escherichia coli* MG1655 with P1 *parS* insertions at different positions and carrying plasmid pALA2705 expressing the GFP-ParB protein (Nielsen *et al.*, 2006a). Cells were grown at 32 °C in AB minimal media supplemented with 0.2% glycerol or 0.2 % glucose, 10 µg/ml uracil and 10 µg/ml thiamin for at least 6 generations and harvested at OD<sub>600</sub> = 0.1

### Software

Image Pro Plus 5.1 was used for all image operations and manual and automatic measurements. The automated counting and measuring procedure was programmed as a macro in Image Pro Plus 5.1.

### Pre-processing

In general pre-processing was not necessary. Basic procedures like subtracting background and dark-current corrections were made. Fluorescent foci could be enhanced by deconvolution, but this did not affect the performance of the macro significantly. Deconvolution and wavelet noise reduction was shown to improve performance on poor quality images however. We preferred to discard such images though, as good quality images were always obtainable with the P1 GFP-ParB/*parS* system.

### Outlining cells on phase contrast images

Outlining of cells was done using the Image Pro Plus 'Count' command. The threshold value was set to  $(B-C)*2/3+C$ , where B is the intensity of the background and C is the average intensity of the cells (in the center). The background intensity was defined as the image

		Automatic count						Performance
Manual count	Foci	Foci	0	1	2	3	4	
	Cells	Cells	3	434	562	7	2	
	0	5	3	2	0	0	0	60.0%
	1	429	0	427	2	0	0	99.5%
	2	564	0	5	559	0	0	99.1%
	3	8	0	0	1	7	0	87.5%
	4	2	0	0	0	0	2	100%

**Table 1.** Automatic counting of foci. The number of cells (grey) with the indicated number of foci (blue) as determined by manual counting is shown in the column to the left. In the upper row the result of counting the same cells automatically is shown. 1008 cells were counted. The central part of the table lists the number of cells in each class (number of foci) where two methods agreed (the diagonal) and the number of cells where they did not. This is summed up in the right column as performance; that is the percentage of cells in each class that the automatic counting counted correctly. The total weighed performance is 99 %.

median intensity value. The average cell intensity was found iteratively:

The cells were outlined using a threshold starting guess and the central intensity for all cells determined and averaged. This average cell intensity was then used to calculate a new threshold value and the cells were outlined again using the new threshold value. This was continued until the average cell intensity value converged and this value was used for the final calculation of the threshold value.

The threshold starting guess was set at the intensity value including  $40 \mu\text{m}^2$  of the image corresponding to the area of approximately 20 cells. On images with less than 20 cells the starting guess will therefore be too high and on images with more than 20 cells it will usually be too low. The following iterations will then find the correct value. This way of determining the initial threshold worked really good and much better than using the Image Pro Plus 'best guess'.

The following filter values were used to sort out non-separable cells and debris: cell area:  $0.8 - 21 \mu\text{m}^2$ , cell aspect ratio:  $1.5 - 10$ , perimeter ratio:  $0.97 - 1$ , cell length:  $1.5 - 6.4 \mu\text{m}$ , cell width:  $0.5 - 1.2 \mu\text{m}$ . These values were appropriate for *E. coli* cells growing in AB glycerol minimal media. For other bacteria and/or other growth media, another set of filter values is needed.

Only fields where the cell contrast,  $(B - C) / B$ , was higher than 0.55 was used, as we found that fields with a smaller contrast value gave more variable results. Often these images turned out to be slightly out of focus or containing dried up cells.

#### *Identifying fluorescent foci*

This is done individually for each cell by defining a proper threshold value of which all foci are brighter and at the same time the background intensity is lower. This threshold value is chosen as the highest one of 7 initial choices (see results) giving the highest number of foci that at the same time meets the following criteria:

1. The foci may not be too variable in size (area). The difference between the largest and smallest focus divided by the mean focus size may not be larger than 1.3.
2. The foci may not be too variable in intensity. The difference between the intensity of the brightest and dimmest focus divided by the intensity of the brightest focus may not be larger than 0.3.
3. The dimmest focus may not be too close to the background intensity. The difference between the intensity of the dimmest focus and the lowest threshold value divided by the lowest threshold value may not be lower than 0.1.

These values were found by trial and error. The center of a focus was defined as the center of gravity,

thus weighing not only the area of the focus but also it's intensity distribution. Each focus' center coordinates, size and intensity is recorded.

#### *Identifying septated and dividing cells*

During the outlining of cells an intensity profile was made for each cell. The intensity profile is a histogram of average intensity values over the length axis of the cell, and is created by slicing the cell in equally sized slices as a sausage and integrating the intensity in each slice (each slice is one pixel or  $0.06 \mu\text{m}$  wide). A septated or dividing cell will have higher intensity values in the central part of the cell because of the invagination of the membrane (the background is brighter than the cell). Each cell were given a score according to the formula  $(I - C)/(B - C)$ , where I is the intensity of the brightest slice in the central third of the cell, C is the cell intensity and B is the image background intensity. Septated cells generally scored between 0.25 and 0.45. Cells very close to division and divided cells scored above 0.45. The 'Line profile' tool of Image Pro Plus 5.1 was used to determine the cell intensity profile. The average of 5 line profiles parallel to the long axis and equally spaced on the cells short axis gave the cell intensity profile.

## References

- Bennett,A., Osterberg,H., Jupnik,H., and Richards,O. (1951) Phase Microscopy: Principles and Applications John Wiley and Sons, Inc., New York, pp. 1-320.
- Gordon,G.S., Sitnikov,D., Webb,C.D., Teleman,A., Straight,A., Losick,R. *et al.* (1997) Chromosome and low copy plasmid segregation in *E. coli*: visual evidence for distinct mechanisms. *Cell* **90**: 1113-1121.
- Koch,A.L. (1996) Similarities and Differences of Individual Bacteria within a Clone. In *Escherichia coli and Salmonella cellular and molecular biology*. Neidhardt,F.C., Ingraham,J.L., Lin,E.C.C., Low,K.B., Magasanik,B., Reznikoff,W.S. *et al.* (eds). Washington D.C.: ASM Press, pp. 1640-1651.
- Lau,I.F., Filipe,S.R., Soballe,B., Okstad,O.A., Barre,F.X., and Sherratt,D.J. (2003) Spatial and temporal organization of replicating *Escherichia coli* chromosomes. *Mol Microbiol* **49**: 731-743.
- Li,Y., Sergueev,K., and Austin,S. (2002) The segregation of the *Escherichia coli* origin and terminus of replication. *Mol Microbiol* **46**: 985-996.
- Nielsen,H.J., Li,Y., Youngren,B., Hansen,F.G., and Austin,S. (2006a) Progressive segregation of the *Escherichia coli* chromosome. *Mol Microbiol* **61**: 383-393.
- Nielsen,H.J., Ottesen,J.R., Youngren,B., Austin,S.J., and Hansen,F.G. (2006b) The *Escherichia coli* chromosome is organized with the left and right chromosome arms in separate cell halves. *Mol Microbiol* **62**: 331-338.
- Niki,H., and Hiraga,S. (1998) Polar localization of the replication origin and terminus in *Escherichia coli* nucleoids during chromosome partitioning. *Genes Dev* **12**: 1036-1045.
- Wang,X., Liu,X., Possoz,C., and Sherratt,D.J. (2006) The two *Escherichia coli* chromosome arms locate to separate cell halves. *Genes Dev* **20**: 1727-1731.

# Progressive segregation of the *Escherichia coli* chromosome

Henrik J. Nielsen,<sup>1</sup> Yongfang Li,<sup>2</sup> Brenda Youngren,<sup>2</sup> Flemming G. Hansen<sup>1\*</sup> and Stuart Austin<sup>2</sup>

<sup>1</sup>BioCentrum-DTU, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark.

<sup>2</sup>Gene Regulation and Chromosome Biology Laboratory, National Cancer Institute, CCR, NCI-Frederick, Frederick, MD 21702-1201, USA.

## Summary

**We have followed the fate of 14 different loci around the *Escherichia coli* chromosome in living cells at slow growth rate using a highly efficient labelling system and automated measurements. Loci are segregated as they are replicated, but with a marked delay. Most markers segregate in a smooth temporal progression from origin to terminus. Thus, the overall pattern is one of continuous segregation during replication and is not consistent with recently published models invoking extensive sister chromosome cohesion followed by simultaneous segregation of the bulk of the chromosome. The terminus, and a region immediately clockwise from the origin, are exceptions to the overall pattern and are subjected to a more extensive delay prior to segregation. The origin region and nearby loci are replicated and segregated from the cell centre, later markers from the various positions where they lie in the nucleoid, and the terminus region from the cell centre. Segregation appears to leave one copy of each locus in place, and rapidly transport the other to the other side of the cell centre.**

## Introduction

Replication of the circular *Escherichia coli* chromosome proceeds bidirectionally from a single origin and terminates on the opposite side. During this process, the daughter chromosomes must be resolved into two separate nucleoid masses despite the fact that they are several orders of magnitude longer than the cell itself, and that replication initially causes them to be linked together by catenation (Zechiedrich and Cozzarelli, 1995).

Once in the bacterial cell cycle, the mass per chromosomal origin reaches a threshold value (the initiation mass; Donachie, 1968) and chromosome replication is initiated at all origins (Skarstad *et al.*, 1986). In slow growing cells, where the replication and post replication periods (C and D) are shorter than the generation time, the bacteria also have a pre-replication period (B) and they initiate chromosome replication from only one origin (Helmstetter, 1996). After termination of chromosome replication, the cell will divide forming two new cells, each with one copy of the chromosome.

Our understanding of chromosome duplication and segregation dynamics has been greatly enhanced by the development of methods for determining the physical positions of specific proteins or DNA sequences in the cell. Loci can be detected by fluorescence in fixed cells by *in situ* hybridization (FISH; Niki and Hiraga, 1998). By using fluorescent techniques such as the GFP-ParB/*parS* system (Li *et al.*, 2002) or the GFP-LacI/operator system (Gordon *et al.*, 1997; Lau *et al.*, 2003), it has become possible to detect the position of discrete DNA loci inside the living cell. Using these techniques, it is possible to determine the movement of individual parts of the chromosome as they are replicated and segregated.

A Factory model for DNA replication and segregation in bacteria has been proposed (Dingman, 1974; Lemon and Grossman, 2000). In this model, the replication machinery is tethered to the cell centre, and the chromosome feeds into it as replication proceeds. The model was recently refined and renamed as the Extrusion-Capture model (Lemon and Grossman, 2001). In this model, the newly replicated DNA is directed away from the cell centre towards the cell poles by the replication process itself. Thus, segregation of sister markers occurs as replication proceeds. This model has some experimental support. Several reports suggest that a replication factory is located in the middle of the cell (Lemon and Grossman, 1998; 2000; Koppes *et al.*, 1999; Lau *et al.*, 2003; Molina and Skarstad, 2004). In addition, origin-proximal loci appear to divide at the cell centre and segregate towards the cell poles (Gordon *et al.*, 1997; Sherratt *et al.*, 2001; Fekete and Chattoraj, 2005).

Other observations have suggested that the Factory model may need major modifications. There have been indications that the two replications forks of the replication factory split up and migrate to the 1/4 and 3/4 positions

Accepted 15 May, 2006. \*For correspondence. E-mail fgh@biocentrum.dtu.dk; Tel. (+45) 45 25 25 05; Fax (+45) 45 93 28 09.



sometime after initiation (the Translocating Replication Apparatuses model) (Niki *et al.*, 2000; Yamazoe *et al.*, 2005). In a radical departure from the Factory model, evidence has been presented that replication produces sister chromosomes that are paired along much of their length. In this Sister Chromosome Cohesion model, most of the chromosomal markers segregate together by an unspecified mechanism later in the cell cycle (Niki *et al.*, 2000; Sunako *et al.*, 2001; Bates and Kleckner, 2005; Yamazoe *et al.*, 2005).

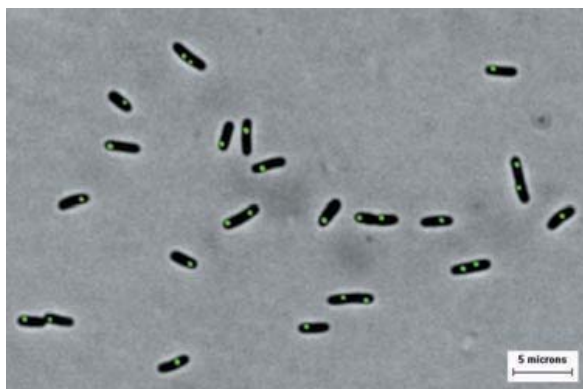
The main objective of our study was to determine the dynamics of chromosome segregation by recording the positions of different chromosomal loci during the cell cycle. We use a refined version of the GFP-ParB/*parS* method to label the loci (Li *et al.*, 2002). Alternative fluorescence labelling methods often give many cells without foci, and the number of fluorescent foci detected appears to be an underestimate. Previous studies using the GFP-ParB/*parS* method were not entirely free from such problems. We have fine-tuned the GFP-ParB/*parS* system to a level where all cells show foci. In addition, we have used a highly efficient, semi-automated detection method that records the number and positions of foci in the cells accurately without observer bias.

We use slow growing cells in order to keep the cell cycle as simple as possible. In the majority of cells in our experiments, a single origin is duplicated during the cell cycle and only one genome equivalent (one completed chromosome) is passed on to daughter cells. To facilitate accurate interpretation of the data, accurate cell cycle parameters were determined for the observed cell cultures, and the cells were alive, in balanced growth, and photographed immediately after the sample was taken. Our results show that segregation is a progressive process, generally following the same program as replication, but with a significant and fairly constant delay. Thus, our results are not consistent with hypotheses involving the simultaneous segregation of the bulk of the chromosome.

## Results

### *Reduced GFP-ParB expression improves the efficiency of detection of marked loci*

The GFP-ParB/*parS* DNA locus detection system depends on the nucleation of GFP-ParB binding to an introduced P1 *parS* sequence, and its spreading to adjacent sequences (Li *et al.*, 2002). In earlier studies, GFP-ParB synthesis was induced from a *lac* promoter with a relatively high concentration of IPTG (100  $\mu$ M; Li *et al.*, 2002). Under these conditions, considerably fewer origin foci were observed in the microscope than the number of origins determined by flow cytometry. Hence some foci

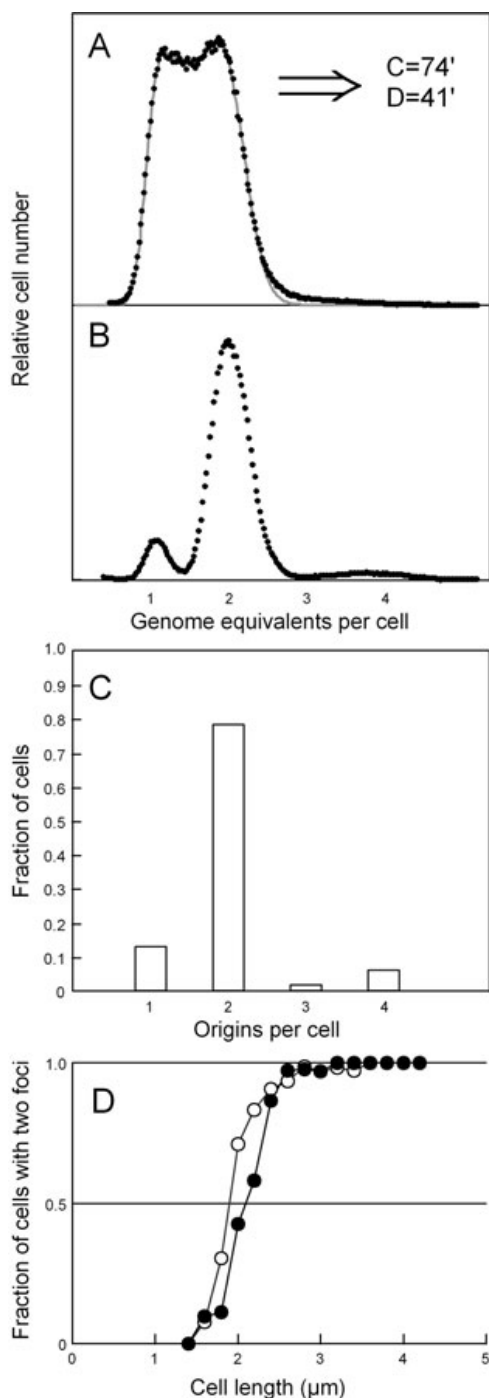


**Fig. 1.** Fluorescence and phase contrast micrograph of slowly growing *E. coli* cells. The fluorescence picture is merged with the phase contrast picture. The cells carried a P1 *parS* sequence localized at 54.2 min on the *E. coli* map and contained plasmid pALA2705 expressing a P1 ParB protein fused to GFP (Li *et al.*, 2002).

were missed, or pairs of foci were observed as single foci. Subsequently, we found that foci were still visible in all cells without induction of the gene encoding GFP-ParB. Using this method the average number of foci per cell increases significantly (data not shown). This was found to be due to two effects. First, the foci become very small and distinct, so that two foci close together are more readily resolved (Fig. 1). This is important, because the limited resolution of the light/fluorescence microscope makes it difficult to resolve daughter foci as they begin to move apart during segregation. Second, limited protein levels prevent pairs of foci sticking together in a fraction of the cells (H.J. Nielsen, unpublished observations). Stickiness is presumably caused by aggregation of excessive levels of bound protein that can, in extreme cases, prevent replicated loci from coming apart. Therefore, the study we present here was carried out at the basal level of expression from the *lac* promoter without IPTG induction.

### *The cell cycle of slow-growing MG1655 cells*

Strain CC4756 is an MG1655 derivative with the P1 *parS* sequence at *attTn7*, close to the origin of replication, and contains a plasmid producing the modified GFP-ParB protein (GFP- $\Delta$ 30ParB; Li *et al.*, 2002). A population of cells was grown in a glycerol minimal medium at 32°C with a generation time of 115 min. The cells were harvested and analysed by flow cytometry (Fig. 2A). Using the software described by Michelsen *et al.* (2003), the C and D periods were estimated to be 71 and 44 min respectively. Hence, initiation of replication occurs close to cell division (Fig. 2A).



#### Detection of marked loci is very efficient

An aliquot of the culture was treated with rifampicin and cephalixin. Under these conditions, rounds of replication complete to produce separate, non-replicating chromosomes. However, the cells stop growing and therefore reflect the size they had reached when sampled (Skarstad *et al.*, 1986). These cells were analysed by flow

**Fig. 2.** Cell cycle parameters. The cell cycle in slowly growing cells was determined by flow cytometry (A). The experimental data (black dots) are compared with a theoretical distribution of genome equivalents (grey line) in a culture with these growth characteristics. The number of origins was determined in a rifampicin- and cephalixin-treated culture by flow cytometry (B) and by fluorescence microscopy (C). The fraction of cells with two (or more) foci in the exponential (closed circles) and the rifampicin-treated culture (open circles) was also determined as a function of cell length (D). Though rifampicin inhibits cell growth, the cells do continue to increase in size for a short time after addition of the drug. The result is a population of slightly larger cells. Thus, the rifampicin-treated cell data were corrected for this residual increase by multiplying the cell sizes by a factor of 0.90 to make the rifampicin-treated cell size distribution identical to the distribution of the non-treated cells.

cytometry (Fig. 2B) and, in addition, by fluorescence microscopy (Fig. 2C). The number and distribution of completed chromosomes, as determined by flow cytometry, was then compared with the number and cell population distribution of fluorescent foci marking the origin. Most of the cells contained two completed chromosomes and had two origin foci in the fluorescent images. A few cells had one or more than two chromosomes, with a corresponding number of cells having one, three or four foci. The comparison of the data in Fig. 2B and C demonstrates a very good correlation between the two detection methods, indicating that almost all origin loci in individual rifampicin-treated cells are well separated and are detected as foci.

The close correspondence of the distributions of foci with the distributions of origins demonstrates that the GFP-ParB/*parS* labelling method is capable of detecting virtually all of the loci in all of the cells as long as the loci are sufficiently well separated. Rifampicin treatment of the cells presumably ensures good separation because all replication is completed to give separate, non-replicating, chromosomes.

#### Origin segregation follows initiation after a delay

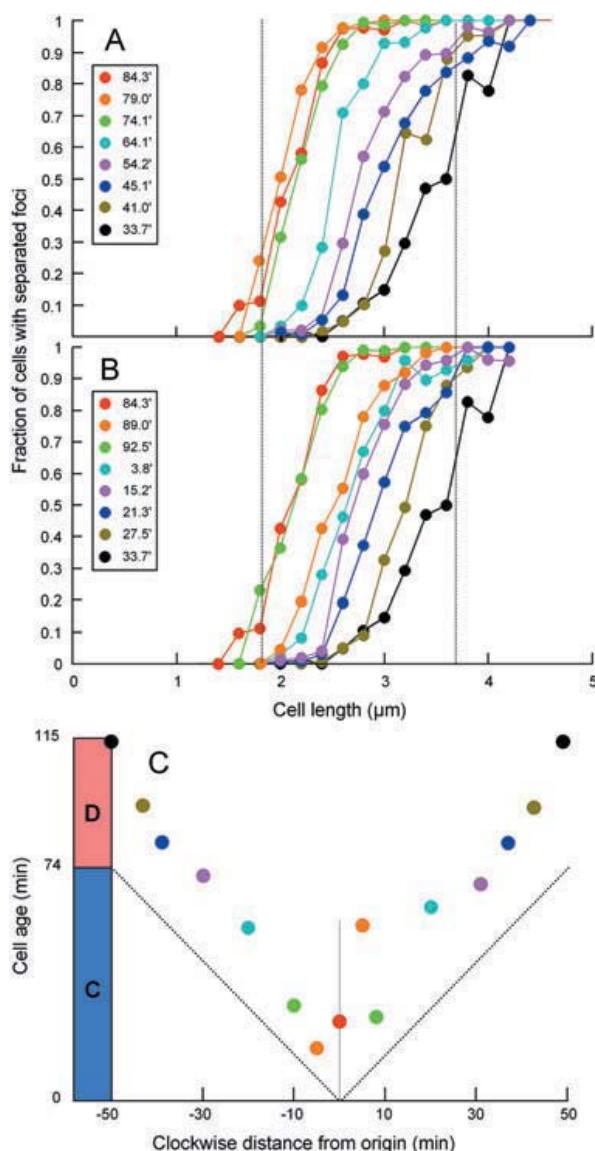
Figure 2D compares the fraction of cells with two foci as a function of cell length from the rifampicin-treated population with that from exponentially growing, non-treated cells. In the rifampicin-treated cells, replication and segregation occur at the same cell size (and thus, at the same apparent cell age) because the cells are not growing as the chromosomes segregate. The distribution of segregation events in the exponential population shows that segregation occurs in slightly larger cells. This effect is presumably due to the time taken for the origin loci to separate after replication and to move sufficiently apart to be detected as two foci. As can be seen from Fig. 2D, this time is equivalent to about 0.2 μm of cell length increase, corresponding to approximately 20% of the cell cycle when converted to cell age.

*Loci are replicated and segregated according to their distance from the origin*

The fraction of cells with two foci, as a function of cell size, was determined in exponentially growing cell populations of 14 strains each with the *parS* sequence inserted at one of 14 different positions on the chromosome. It should be emphasized that flow cytometry showed that these strains all have almost identical cell cycles (identical C and D periods) to that of the origin-labelled strain CC4756 (data not shown). Figure 3 shows that two foci appear in the 14 strains after replication of the respective *parS* sequences in a relatively orderly fashion. The timing of segregation is related to the distance of the locus to the origin of replication. Each locus is segregated after its predicted time of replication with a short delay. This delay is very similar for most loci, so that they are separated in the order in which they are replicated (Fig. 3C). The delay does not result in a large portion of the chromosome separating as a unit, as would be the case in the sister chromosome cohesion model. The chromosomes separate progressively. However, there are some interesting deviations from this general rule. The two foci formed after replication of the *parS* sequence at the 89.0 min locus appear to separate much later than expected from the behaviour of the other loci. This is also true to a lesser extent for the origin locus at 84.2 min. Hence the 79.0 min locus, the marker nearest to the origin in the anticlockwise direction is actually the first to segregate (Fig. 3C). In addition, the terminus segregates considerably later than its projected replication time. Segregation of the terminus is generally delayed until just before cell division (Fig. 3C), as has been shown previously (Li *et al.*, 2002; Lau *et al.*, 2003).

*DNA loci are broadly distributed in the cells*

We looked at the location of the individual fluorescent DNA foci in the cells with respect to the progression of the cell cycle (the length of the cells). The scatter plots in Fig. 4 show that the positions of the foci in individual cells are rather broadly scattered, although, as we will show, they follow some general trends. Some of the scatter is due to measurement errors. Due to the limits of the resolution, an error in determining the centre of a focus of just one pixel will shift its relative position by ~5% for a newborn cell. Also, some scatter results from using cell length as measure of progression through the cell cycle because all cells do not initiate DNA replication at the same length. The size distribution of initiating cells has a coefficient of variation of 15% as judged by flow cytometry (data not shown). This is comparable to the normally observed variability of a cell culture (Koch, 1996). However, much of the scatter does reflect real variations in the positions of foci in individual cells. Foci can move rapidly from one position to another.



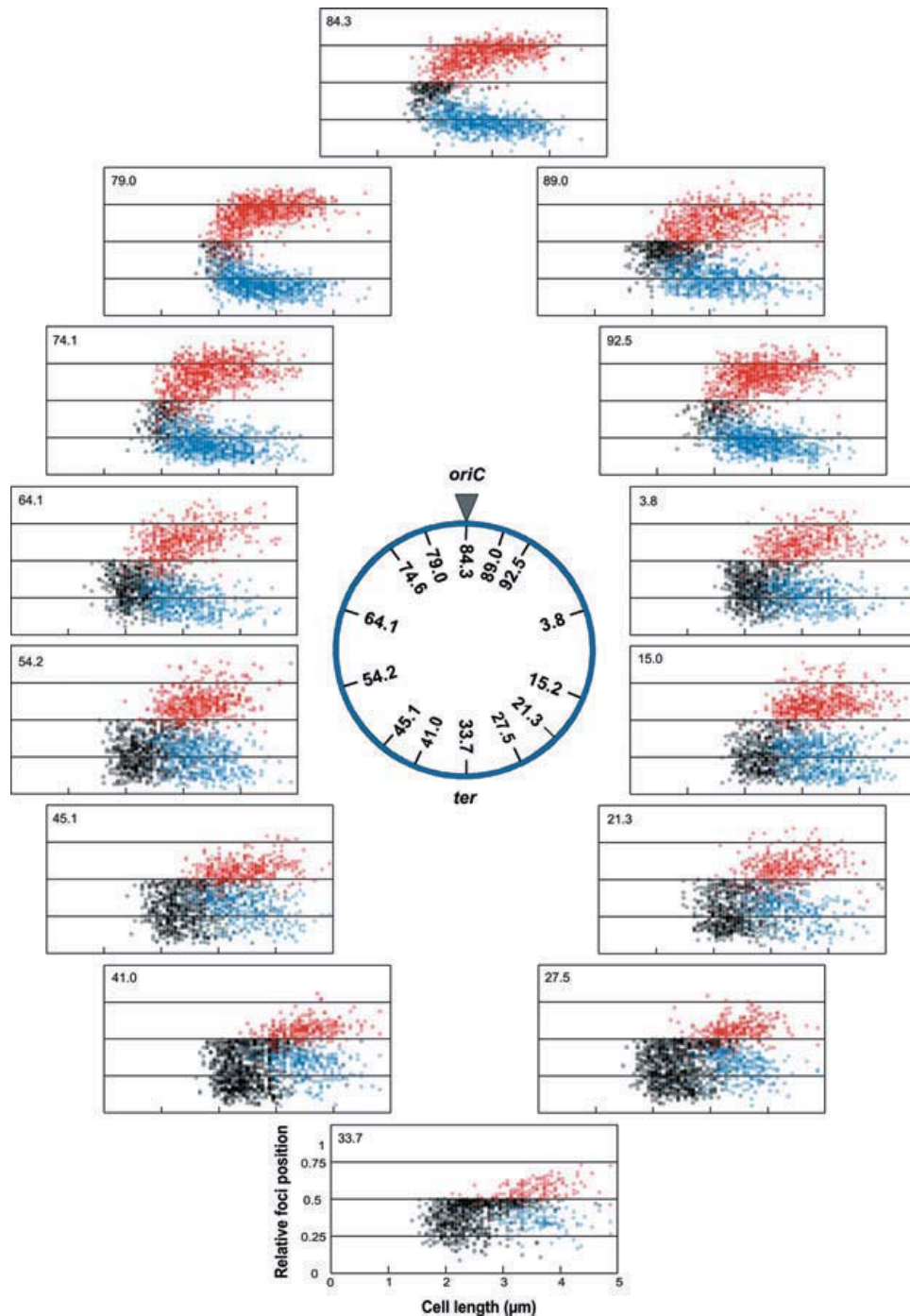
**Fig. 3.** Separation of foci. The fraction of cells with two (or more) foci was determined for all loci with respect to cell length and is shown in panel A (loci clockwise from the origin) and B (counterclockwise loci). The dotted lines in panels A and B represent the average cell size at birth and division respectively. Panel C shows the difference between the expected time of replication, as determined by flow cytometry (dotted lines), and the time of separation, as determined from the separation curves. The time of separation is defined as the cell length, converted to cell age, at which 50% of the cells have separated foci.

We have observed foci moving as much as 0.5  $\mu\text{m}$  in a matter of seconds (Fig. 5).

*Foci follow general positional patterns*

Though the distributions of foci are broad, the average focus position in the cell does seem to follow specific





**Fig. 4.** Positions of chromosomal loci in the cells. The relative positions of foci are presented for cells with the P1 *parS* at the indicated positions on the chromosomal map (centre panel). Foci from cells with one focus are represented by black dots showing the distance to the nearest pole, foci from cells with two foci are represented with a blue dot (the focus which is closest to a pole) and a red dot. Foci from cells with three or four foci are not shown. They constitute 9% of the cells for the 79.0 locus, between 3.8% and 4.6% for the 74.1, 84.3 and 92.5 loci, approximately 1% for the 89.0 locus, and considerably less than 1% for the remaining loci. Note that, for intermediate loci, the lower focus (that is the focus that is closest to a cell pole) shows greater variation than the second focus. This asymmetry is due to a bimodal distribution, with most foci lying within the cell quarters, but some passing beyond the mass of the origin-proximal DNA at the cell quarters. Hence, there are a number of cells with one focus in the central region and one near a pole (see examples in Fig. 1). In all 14 cases the data from 1000 cells have been presented.



**Fig. 5.** Foci are highly mobile. Combined phase contrast and fluorescence images of the same cell taken 1.5 s apart. The cell had the P1 *parS* sequence at 54.2 min on the chromosome.

trends. Inspection of Fig. 4 shows that the scatter patterns of the loci change progressively from origin to terminus. Prior to segregation (black dots), the origin loci tend to cluster close to the cell centre. The further the unreplicated markers are from the origin, the more broadly scattered they become. For the replicated and segregated loci (red or blue dots), the origin-proximal markers are relatively narrowly distributed, and cluster around the cell quarter positions at late times in the cell cycle. The further the segregated markers are from the origin, the more broadly scattered they appear and the closer they tend to lie to the cell centre. Note that the great majority of cells with two foci have one in each half of the cell, irrespective of where the marker is placed. This is true of all loci, with less than 3% of the total two-focus cells having both in one cell half. Moreover, the majority of these 3% have one focus very close to the measured centre, where its position relative to the true cell centre is in doubt (data not shown). Thus, segregation places different loci in different general positions, according to their distance from the origin, but always places duplicate loci in opposite cell halves. Despite their local mobility, foci, once segregated, seldom if ever move back across the cell centre.

#### *From where do the foci segregate?*

Replication is initiated close to cell division in the average cell and we find the average position of the origin foci at the centre of the cell at cell birth (Fig. 4). As the cell cycle progresses, two origin foci move apart to positions at, or close to, the cell quarter positions. The quarter positions will become the centres of the newborn cells after replication. Thus the origins are now positioned correctly for the next generation. Some of the loci close to *oriC* behave similarly (e.g. 79.0 min, Fig. 4). Here we also find cells with a single focus close to the middle of the cell and upon replication they move to positions near the cell quarters. In some cases, these foci move further out than the origin foci. The terminus also appears to be replicated at the cell centre. Following division, and coming from the new cell pole, it is located outside the cell centre at cell birth, but soon migrates to the centre where it remains up to and beyond termination (Fig. 4).

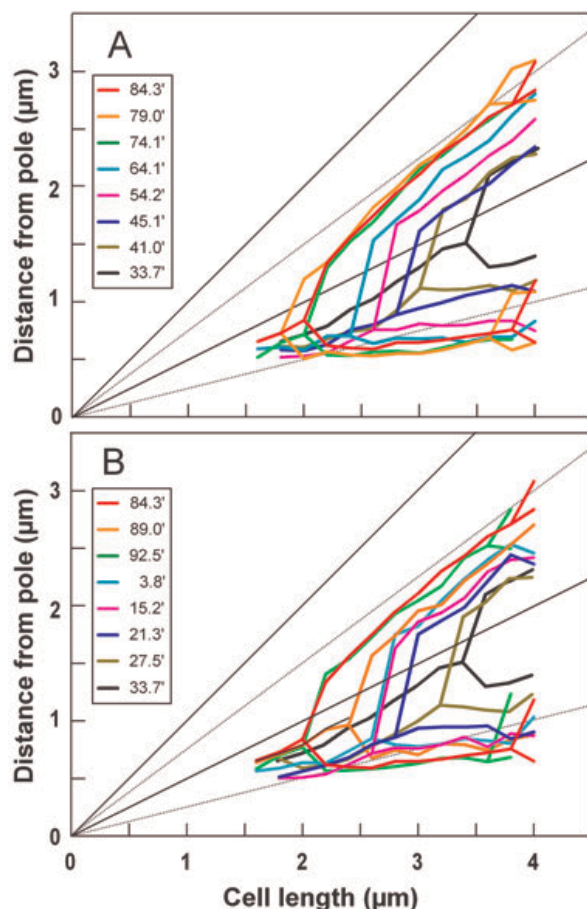
Although the origin and its associated markers are replicated from the cell centre, or close to it, the six intermediate markers (those at 3.8, 15.2, 21.3, 45.1, 54.2 and 64.1 min) show no obvious tendency to go to the centre before replication and segregation (Fig. 4). As there is always a marked delay between replication and segregation where the two newly replicated sister loci stay together and thus appear as one focus which is located away from the centre position, we can rule out the possibility that these foci move to the centre and replicate there. Thus, it appears that the markers are replicated in this non-central position and segregate from there, with one locus staying and the other moving across the cell centre (see below).

#### *The path of segregation*

In Fig. 6 we have combined the separation data in Fig. 3 with the average positions of the foci as calculated from the data in Fig. 4. In these plots, the average point of focus separation is displayed as well as the positioning of the average foci as cell age increases (see Fig. 6 legend). Due to the method used for orienting the cells (see Fig. 6 legend) these plots skew all the data points off-centre. However, they reveal trends in the timing and general path of segregation. The origin-proximal foci separate early at, or near to the cell centre. After initial separation they continue to move slowly outward until they occupy the cell quarter positions. This is probably due to the deposition of the bulk of the newly replicated DNA on the inner faces of the developing nucleoids. This would cause the early markers to be placed, on average, nearer to the poles than later markers. Intermediate loci are segregated later, in the order of their replication, and from average positions that are away from the centre. They move rapidly to characteristic average positions, such that the closer the locus is to the origin, the further the foci tend to migrate towards the pole.

It is interesting to note that, especially in the case of the intermediate loci, one focus tends to remain in the position of its parent whereas its sister moves rapidly to the other side of the cell (Fig. 6). This phase is followed by a small and slow outward movement. This may give space to the loci that are segregated later; or perhaps may reflect the loci being pushed outward by DNA that is subsequently placed there by the segregation process. This is actually very similar to results obtained with time-lapse microscopy of *Caulobacter crescentus* (Viollier *et al.*, 2004).

The replicated terminus foci generally separate late in the cell cycle from the cell centre and segregate in a symmetrical fashion to positions well within the cell quarters. This segregation presumably reflects the positioning of the terminus on the inner face of the dividing



**Fig. 6.** Migration of foci. The figure shows the position and migration of the different loci before and after separation. For each cell size class we determined which class of cells (one focus or two foci) predominated for the loci shown. The average position of these foci was then plotted for that cell size. As all measurements are taken from the cell pole nearest to a focus, the patterns are skewed off-centre towards the lower part of the graph. However, despite this apparent distortion, this is a simple and comprehensive way to show the general behaviour of the chromosomal loci. Cell poles and the midcell positions are indicated by solid diagonal lines. Cell quarters are indicated by grey diagonal lines.

nucleoid, and the subsequent movement of the nucleoids away from each other as the cell prepares to divide.

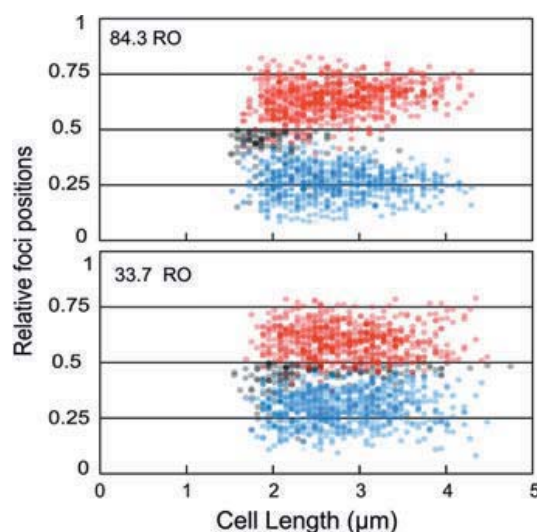
#### Capture of the origin

When we look at the variation of focus positions late in the cell cycle for the origin and other loci (Fig. 4), the distribution of the origin positions in the cells is narrower than the distribution of foci in the strains with the *parS* sequence in other positions. This indicates that the origin is captured at the cell quarters which become the cell centres in readiness for the next round of replication. This hypothesis is supported by data from rifampicin/cephalexin-treated cells. Here, the origin foci in cells in the

later phases of the cell cycle also show a clear tendency to cluster around the cell quarters (Fig. 7). While the foci in cells in early stages of replication show much more scatter. Presumably the cells late in the cell cycle had already attached the origins at the quarter position before the drugs were added. Thus, rifampicin blocks early capture of the origin at the sites of initiation possibly because a protein required for origin capture is missing when the transcription is blocked by rifampicin. In contrast, rifampicin and cephalexin treatment of the strain having the *parS* sequence close to the terminus (33.7) results in distribution of the replicated termini to the two cell halves but they do not show any sign of going to specific positions (Fig. 7).

#### Discussion

We have refined the GFP-ParB/*parS* system by lowering the induction of the GFP-ParB protein. All cells still have visible foci (more than 99%) and these foci are in general more discrete and better separated than previously reported. Moreover a previous problem of pairs of *parS* loci sticking together resulting in the observation of fewer foci than expected is also solved by the lowering of the GFP-ParB protein level in the cell. Although we cannot rule out residual effects of this method of detection on the



**Fig. 7.** Positions of chromosomal loci in cells with terminated replication rounds. The relative positions of foci are presented for cells with the P1 *parS* at the indicated positions. The cultures have been treated with rifampicin and cephalexin to allow run-off (RO), i.e. termination of ongoing rounds of chromosome replication and to stop cell division. Foci from cells with one focus are represented by black dots showing the distance to the nearest pole, foci from cells with two foci are represented with a blue dot (the focus which is closest to a pole) and a red dot. Foci from cells with three or four foci are shown as grey dots.

behaviour of the DNA, the cells grow normally under the conditions that we use, and the comparison of flow cytometry and fluorescence microscopy shown in Fig. 2 shows that chromosome segregation is completed normally in a majority of the cells. Combined with our automated counting method that makes it possible to measure large numbers of cells in a very short period of time, we believe that our system gives a more accurate and detailed picture of how the *E. coli* chromosome is segregated than has previously been possible.

Our results clearly show that most of the chromosome segregates progressively as it replicates. Only the behaviour of the 89' region and the terminus deviates from this pattern. It is conceivable that a specific locus that affects chromosome segregation (*migS*, 89 min; Yamaichi and Niki, 2004; Fekete and Chatteraj, 2005) is involved in the delayed segregation of the 89 min region. The affected chromosome segment (extending clockwise from the 84 min origin through 89 min but stopping short of the 92.5 marker) might be held back at the site of replication by local sister cohesion or affinity to some central structure such as the replication complex. Later markers on the same half of the chromosome conform to the general pattern and are segregated in the order of replication. All markers on the other chromosome arm segregate smoothly and progressively in their order of replication. Thus, we find no evidence for extensive sister chromosome cohesion and simultaneous segregation of the bulk of the chromosome as has recently been proposed (Sunako *et al.*, 2001; Bates and Kleckner, 2005).

Bates and Kleckner (2005) reported a study of chromosome segregation using FISH. They studied the segregation of the origin and terminus and two intermediate markers on the clockwise arm of the chromosome. The two markers, *glnA* (87.3 min) and *lac* (7.8 min), segregated at approximately the same time. From this they concluded that, following replication, an extensive portion of the clockwise half of the sister chromosomes are paired by sister cohesion and segregate as a unit some time later. They assumed that the anticlockwise half of the chromosome behaves similarly. Our work provides an alternative explanation. The *glnA* locus is close to the 89 min region that is subjected to an extended delay for segregation while the *lac* marker is well beyond it. Thus, their segregation at approximately the same time appears to be coincidental (cf. the 89 min locus versus the 3.8 min locus in our Fig. 3B).

Although the segregation of the new chromosomes takes place continuously, there is a fairly constant delay of about 20% of the cell cycle between replication and visible segregation for most markers (Fig. 3C). This delay is unlikely to be due solely to our inability to resolve closely spaced foci. It appears that the newly synthesized loci are held in the vicinity of the forks before segregation. Perhaps

the homologues are held together for a while after replication. This might involve SeqA molecules that bind the hemi-methylated GATC sequences on the newly replicated DNA (Guarne *et al.*, 2005). This temporary cohesion of sister loci might give the replication/repair system time to do any necessary homologous recombination before the DNA strands are segregated. A delay of this magnitude makes it less likely that the activity of the polymerase at the forks provides the direct motive force for segregation. Rather it appears that other forces, which may include passive dispersal and condensation, ultimately drive segregation (Norris *et al.*, 2004). An active process to initiate segregation involving the directional transport of the origin regions cannot, however, be ruled out.

The intermediate markers of both arms of the chromosome appear to replicate and segregate from a variety of positions in the cell. The markers are broadly distributed before replication. After replication, during the delay prior to segregation, they remain as single foci in the same pattern. Thus, replication appears to occur at the original location of the marker, and the two copies remain close to each other at the site of synthesis. Segregation then occurs by a mechanism that leaves one copy in place, and rapidly moves the other to the opposite cell half. These results are consistent with independent travel of the forks around the chromosome arms, rather than with a permanent central factory for replication. This is in agreement with the studies of Bates and Kleckner (2005) on the behaviour of DnaX-GFP foci formed by the replicative polymerase, and the studies of Yamazoe *et al.* (2005) on the behaviour of SeqA foci which form on newly replicated DNA at the replication forks.

To summarize our findings: at initiation, the nucleoid has the origin at or near the cell centre but does not necessarily have any fixed position for other markers excepting that origin-proximal markers tend to be nearer the centre and terminus-proximal ones are broadly distributed out towards the cell poles. Initiation occurs at the centre and newly replicated DNA for the origin region begins to accumulate there. After a delay, lasting approximately 20% of the cell cycle, the replicated origin regions are separated and move outward. The intermediate loci on both sides of the chromosome are broadly scattered and are segregated in a delayed fashion as are the loci close to the origin, i.e. the two replicated loci stay together for some time at the position the unreplicated locus occupied. The delay of segregation of the different loci, we define as a sister loci cohesion period. This is different from the notion of sister chromosome cohesion where a major part of the chromosome is segregated as a unit. If this was case, the points in Fig. 3C would be positioned on a horizontal line. Thus, we suggest that as replication proceeds beyond the origin region, the forks leave the centre and follow the path of the DNA arms. As each



marker is replicated, the two copies appear to stay in place for some time, before the other rapidly moves across the cell mid-line, adding to the bulk of the other nascent nucleoid. Most of the added DNA presumably accumulates inside the previously replicated DNA, pushing earlier markers progressively outward. This would explain the slow progression of the early markers outward, and why, on average, earlier markers lie closer to the cell poles than later ones (Fig. 6). When the origin reaches the cell quarter position, it appears to be captured there. Other markers lie roughly in the positions defined by their order of replication. Presumably, the transported markers are added to the DNA mass in an orderly fashion, but considerable mixing of the DNA regions occurs as the new nucleoids are created, thus explaining the broad scatter of intermediate markers in Fig. 4 at all phases of the cell cycle. We note that the distributions of replicated intermediate markers are not continuous. These loci tend to be excluded from the cell quarter positions (Fig. 4). This may be due to exclusion by the mass of the origin-proximal DNA that is tethered by capture at the cell quarters. As replication completes, the outward trend of the motion of the forks must be interrupted, because the terminus is clearly held at the cell centre at this stage, presumably by association with the FtsK protein (Liu *et al.*, 1998; Yu *et al.*, 1998; Corre and Louarn, 2005). Thus the forks must move to the cell centre again as the terminus is replicated and the two completed sister nucleoids come apart.

Based on the localization of markers near the terminus, Wang *et al.* (2005) have suggested that the resting nucleoid may have the origin at the centre and the two arms arranged with one on one side of the cell and one on the other. If markers on the left and right arms of the chromosome are randomly placed within the nucleoid, the forks would have to cross back and forth across the cell centre-line, and would often both be on the same side of the cell. Based on the distribution of SeqA foci, this is not the case (Brendler *et al.*, 2000; Yamazoe *et al.*, 2005). Thus, it seems probable that the left and right arms of the chromosome are primarily located in opposite cell halves, similarly to what has been observed for the *Bacillus subtilis* chromosome (Teleman *et al.*, 1998). The 'one copy moves and one stays' mechanism would perpetuate this arrangement, and would produce sister nucleoids whose general pattern of marker placement would be as shown and discussed here and as suggested by Wang *et al.* (2005).

## Experimental procedures

### Bacterial strains and media

The strains used were derivatives of *E. coli* MG1655 with P1*parS* inserted at 14 different positions around the chromosome. The *parS* sequences were initially inserted into

strain DY330 as described by Li *et al.* (2002), using the recombineering technique (Yu *et al.*, 2000) with the oligonucleotides listed in *Supplementary material* (Table S1). The marked loci were then transferred to strain MG1655 by P1 transduction, selecting for the kanamycin-resistance gene that is linked to each *parS* insert. The GFP- $\Delta$ 30ParB protein was expressed from pALA2705 (Li *et al.*, 2002). Strains were grown exponentially for at least six generations before samples were taken for microscopy.

### Microscopy

Cells were grown at 32°C in AB minimal media (Clark and Maaløe, 1967) with 0.2% glycerol supplemented with 1  $\mu\text{g ml}^{-1}$  thiamine, 1  $\mu\text{g ml}^{-1}$  uracil and 100  $\mu\text{g ml}^{-1}$  ampicillin. From the growing culture, 1 ml was harvested, concentrated 50-fold, and 2  $\mu\text{l}$  was placed on a polylysine-coated glass slide, covered with a coverslip, and cells immobilized by pressing the coverslip with the thumb. As the cells applied to the microscope slide are living, the cell population is expected to drift out of balanced growth with time on the slide. However, when comparing populations that had been on the slide for the minimum practical amount of time (90 s) with those left on the slide for an extended period approximating the maximum length of time incurred in our experiments (10 min) we found no significant differences between the two populations. Microscopy was carried out on a Nikon Eclipse E-1000 microscope equipped with a Nikon C-CU Universal condenser, a Nikon Plan Fluor 100 $\times$  objective and a Hamamatsu Orca-ER c4742-95 CCD camera. Images were acquired using Openlab 4.2 software.

### Automatic measurement of cells and foci

The length of each cell, the number of foci, and the position of the foci centres relative to the long cell axis were measured automatically using the Image Pro Plus 5.1 image analysis program. The relevant features of the program were linked by macro programming to adapt them to the specific task in hand.

### Flow cytometry

Cells were either harvested directly from the exponentially growing culture (exponential sample) or a sample was taken to a container containing the minimal media previously described supplemented with rifampicin and cephalixin to a final concentration of 200  $\mu\text{g ml}^{-1}$  and 36  $\mu\text{g ml}^{-1}$ , respectively, to terminate ongoing rounds of replication and stop cell division and incubated for 5 h at 32°C and stop cell division. Cells were washed twice in buffer (1 mM EDTA, 10 mM Tris HCl pH 7.4), fixed in 70% ethanol and stored at 5°C. Before the analysis in the flow cytometer, the cells were washed once in 1 ml of 0.01 M  $\text{MgCl}_2$ , 0.01 M Tris pH 7.5. The cells were centrifuged and the pellet suspended in 100  $\mu\text{l}$  of buffer. Mithramycin A and ethidium bromide was added to final concentrations of 100  $\mu\text{g ml}^{-1}$  and 20  $\mu\text{g ml}^{-1}$  respectively. The cells were left at 4°C in the dark for 1 h. The cell size and DNA content distributions were determined in a flow cytometer.

eter (Bryte SH, Bio-Rad, Hercules, CA) equipped with a 100 W Osram mercury short-arc HBO lamp.

## Acknowledgements

We thank Tove Atlung for discussions and comments on the manuscript. The *trans*-atlantic cooperation between the two laboratories was made possible by funding from the Otto Mønsted foundation and the Oticon foundation in Denmark.

## References

- Bates, D., and Kleckner, N. (2005) Chromosome and replisome dynamics in *E. coli*: loss of sister cohesion triggers global chromosome movement and mediates chromosome segregation. *Cell* **121**: 899–911.
- Brendler, T., Sawitzke, J., Sergueev, K., and Austin, S. (2000) A case for sliding SeqA tracts at anchored replication forks during *Escherichia coli* chromosome replication and segregation. *EMBO J* **19**: 6249–6258.
- Clark, D.J., and Maaløe, O. (1967) DNA replication and the division cycle in *Escherichia coli*. *J Mol Biol* **23**: 99–112.
- Corre, J., and Louarn, J.M. (2005) Extent of the activity domain and possible roles of FtsK in the *Escherichia coli* chromosome terminus. *Mol Microbiol* **56**: 1539–1548.
- Dingman, C.W. (1974) Bidirectional chromosome replication: some topological considerations. *J Theor Biol* **43**: 187–195.
- Donachie, W.D. (1968) Relationship between cell size and time of initiation of DNA replication. *Nature* **219**: 1077–1079.
- Fekete, R.A., and Chatteraj, D.K. (2005) A cis-acting sequence involved in chromosome segregation in *Escherichia coli*. *Mol Microbiol* **55**: 175–183.
- Gordon, G.S., Sitnikov, D., Webb, C.D., Teleman, A., Straight, A., Losick, R., et al. (1997) Chromosome and low copy plasmid segregation in *E. coli*: visual evidence for distinct mechanisms. *Cell* **90**: 1113–1121.
- Guarne, A., Brendler, T., Zhao, Q., Ghirlando, R., Austin, S., and Yang, W. (2005) Crystal structure of a SeqA-N filament: implications for DNA replication and chromosome organization. *EMBO J* **24**: 1502–1511.
- Helmstetter, C.E. (1996) Timing of synthetic activities in the cell cycle. In *Escherichia coli and Salmonella Cellular, Molecular Biology*. Neidhardt, F.C., Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., et al. (eds). Washington, DC: American Society for Microbiology Press, pp. 1627–1639.
- Koch, A.L. (1996) Similarities and differences of individual bacteria within a clone. In *Escherichia coli and Salmonella Cellular, Molecular Biology*. Neidhardt, F.C., Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., et al. (eds). Washington, DC: American Society for Microbiology Press, pp. 1640–1651.
- Koppes, L.J., Woldringh, C.L., and Nanninga, N. (1999) *Escherichia coli* contains a DNA replication compartment in the cell center. *Biochimie* **81**: 803–810.
- Lau, I.F., Filipe, S.R., Soballe, B., Okstad, O.A., Barre, F.X., and Sherratt, D.J. (2003) Spatial and temporal organization of replicating *Escherichia coli* chromosomes. *Mol Microbiol* **49**: 731–743.
- Lemon, K.P., and Grossman, A.D. (1998) Localization of bacterial DNA polymerase: evidence for a factory model of replication. *Science* **282**: 1516–1519.
- Lemon, K.P., and Grossman, A.D. (2000) Movement of replicating DNA through a stationary replisome. *Mol Cell* **6**: 1321–1330.
- Lemon, K.P., and Grossman, A.D. (2001) The extrusion-capture model for chromosome partitioning in bacteria. *Genes Dev* **15**: 2031–2041.
- Li, Y., Sergueev, K., and Austin, S. (2002) The segregation of the *Escherichia coli* origin and terminus of replication. *Mol Microbiol* **46**: 985–996.
- Liu, G., Draper, G.C., and Donachie, W.D. (1998) FtsK is a bifunctional protein involved in cell division and chromosome localization in *Escherichia coli*. *Mol Microbiol* **29**: 893–903.
- Michelsen, O., Teixeira de Mattos, M.J., Jensen, P.R., and Hansen, F.G. (2003) Precise determinations of C and D periods by flow cytometry in *Escherichia coli* K-12 and B/r. *Microbiology* **149**: 1001–1010.
- Molina, F., and Skarstad, K. (2004) Replication fork and SeqA focus distributions in *Escherichia coli* suggest a replication hyperstructure dependent on nucleotide metabolism. *Mol Microbiol* **52**: 1597–1612.
- Niki, H., and Hiraga, S. (1998) Polar localization of the replication origin and terminus in *Escherichia coli* nucleoids during chromosome partitioning. *Genes Dev* **12**: 1036–1045.
- Niki, H., Yamaichi, Y., and Hiraga, S. (2000) Dynamic organization of chromosomal DNA in *Escherichia coli*. *Genes Dev* **14**: 212–223.
- Norris, V., Woldringh, C., and Mileyskaya, E. (2004) A hypothesis to explain division site selection in *Escherichia coli* by combining nucleoid occlusion and Min. *FEBS Lett* **561**: 3–10.
- Sherratt, D.J., Lau, I.F., and Barre, F.X. (2001) Chromosome segregation. *Curr Opin Microbiol* **4**: 653–659.
- Skarstad, K., Boye, E., and Steen, H.B. (1986) Timing of initiation of chromosome replication in individual *E. coli* cells. *EMBO J* **5**: 1711–1717.
- Sunako, Y., Onogi, T., and Hiraga, S. (2001) Sister chromosome cohesion of *Escherichia coli*. *Mol Microbiol* **42**: 1233–1241.
- Teleman, A.A., Graumann, P.L., Lin, D.C., Grossman, A.D., and Losick, R. (1998) Chromosome arrangement within a bacterium. *Curr Biol* **8**: 1102–1109.
- Viollier, P.H., Thanbichler, M., McGrath, P.T., West, L., Meewan, M., McAdams, H.H., and Shapiro, L. (2004) Rapid and sequential movement of individual chromosomal loci to specific subcellular locations during bacterial DNA replication. *Proc Natl Acad Sci USA* **101**: 9257–9262.
- Wang, X., Possoz, C., and Sherratt, D.J. (2005) Dancing around the divisome: asymmetric chromosome segregation in *Escherichia coli*. *Genes Dev* **19**: 2367–2377.
- Yamaichi, Y., and Niki, H. (2004) *migS*, a cis-acting site that affects bipolar positioning of *oriC* on the *Escherichia coli* chromosome. *EMBO J* **23**: 221–233.
- Yamazoe, M., Adachi, S., Kanaya, S., Ohsumi, K., and Hiraga, S. (2005) Sequential binding of SeqA protein to nascent DNA segments at replication forks in synchronized cultures of *Escherichia coli*. *Mol Microbiol* **55**: 289–298.

- Yu, D., Ellis, H.M., Lee, E.C., Jenkins, N.A., Copeland, N.G., and Court, D.L. (2000) An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc Natl Acad Sci USA* **97**: 5978–5983.
- Yu, X.C., Tran, A.H., Sun, Q., and Margolin, W. (1998) Localization of cell division protein FtsK to the *Escherichia coli* septum and identification of a potential N-terminal targeting domain. *J Bacteriol* **180**: 1296–1304.
- Zechiedrich, E.L., and Cozzarelli, N.R. (1995) Roles of topoisomerase IV and DNA gyrase in DNA unlinking during replication in *Escherichia coli*. *Genes Dev* **9**: 2859–2869.

## Supplementary material

**Table 1. Oligomer sequences**

Oligonucleotides used for chromosomal insertions of the *parS* site into the genome of MG1655. The underlined sequence is the sequence that is homologue with the chromosomal insertion position; the rest is the sequence homologue to the template containing the *parS*.

Insert position	Primer 1	Primer 2
84.3 min - proximal AttTn7	5' - GCA GGA TGT TTG ATT AAA AAC <u>ATA ACA GGA GAA AAA TGC CGA TAA</u> AAA GCC GAA GCC TTA AAC	5' - AAT CGG TTA CGG TTG AGT AAT <u>AAA TGG ATG CCC TGC GTA ACA CAG</u> CTT TAG AGC GTT TTG CGA T.
33.7 min - 20 kb to dif.	5' - GCA GGG TAT TGC CCA ACA GAA <u>CAG CTT TAA ACA TAC CTG ACG ATA</u> AAA AGC CGA AGC CTT AAA C	5' - TGA GCT GCT TAG CTT TAC CTG <u>TCT GTA CTG ATG TAG CCA TCA CAG</u> CTT TAG AGC GTT TTG CGA T
15.0 min	5' - GAT CGT CCA TTT TCT TGA ACG <u>CTT CAT CCC ATT CGA TCG CCG ATA</u> AAA AGC CGA AGC CTT AAA C	5' - AGT TGA GCT GCG TAA GAA AGC <u>CCT CGA GCT GTC ACG CCT GCA CAG</u> CTT TAG AGC GTT TTG CGA T
21.3 min	5' - ACG TGG TGA ATC TGA AAC TCG <u>CCT GAG AAA ACA GGG GTA ACG ATA</u> AAA AGC CGA AGC CTT AAA C	5' - TGG CGA TCT GTA CGA GAA GAT <u>AAC TAA AGC ACT GGC TTG ACA CAG</u> CTT TAG AGC GTT TTG CGA T
3.8 min	5' - GGC GCG AAG CAA GGC CGC ATC <u>AGC CAG CGA GAA TAC TTG ACG ATA</u> AAA AGC CGA AGC CTT AAA C	5' - CGT CGG TAA ACT CCA GCG GCA <u>GTG CTA CGT CAT CAC TCA TCA CAG</u> CTT TAG AGC GTT TTG CGA T
45.1 min	5' - GAC TAT TAC CGA TTT AGC GCG <u>TGA AAA CGT CCG CAA CCT GCG ATA</u> AAA AGC CGA AGC CTT AAA C	5' - CAC GTA AGG CGT CAA TGA CGC <u>GCT GGC TTT CTT CAC GGG TCA CAG</u> CTT TAG AGC GTT TTG CGA T
54.2 min	5' - CAA TTT CCT CGC CCG TCT CGA <u>TAT TGA CGA ATC AGC ATA ACG ATA</u> AAA AGC CGA AGC CTT AAA C	5' - AGC GCT GGA TTT TAT TGC TGA <u>ACG CGA AAA TCA GCA GTA ACA CAG</u> CTT TAG AGC GTT TTG CGA T
64.1 min	5' - CGA TAC CGA TCT CAC CGC CGA <u>AAA TCT GCT GCG TTT GCC CCG ATA</u> AAA AGC CGA AGC CTT AAA C	5' - CTT CGA TGG TCT GGC GAC GGC <u>GAA TCA ACC GCG CCT GAC CCA CAG</u> CTT TAG AGC GTT TTG CGA T
74.1 min	5' - TTA CTT CCG CCA TTT TTG CCA <u>GCT CAC CAA TGC GAT ACA TCG ATA</u> AAA AGC CGA AGC CTT AAA C	5' - TTA TGC TGA CAA GTT GTT GGA <u>CAA AAT CAA CGA TAA CTA GCA CAG</u> CTT TAG AGC GTT TTG CGA T
79,0 min	5' - GCA AAC CGC CCG CTA AAA GTC <u>GAA TTT TTG TGC CCT GCA TCG ATA</u> AAA AGC CGA AGC CTT AAA C	5' - TCG TGC GCC GGA TGG CAA AAC <u>GCA CGA ATT ATC CTC TTA ACA CAG</u> CTT TAG AGC GTT TTG CGA T
89,0 min	5' - CAG TGG CTG TGG TGT TAT GGA <u>TAT CGT CTG ACG TGC TCA TCG ATA</u> AAA AGC CGA AGC CTT AAA C	5' - CGC GAT TTG CCA TAC GCT GGG <u>GGT TCG ACC TGG TTT ATA ACA CAG</u> CTT TAG AGC GTT TTG CGA T
92.5 min	5' - CAA GTT GAA AAC TCG CCT GCG <u>CGA AGC GGC ACT GGC GTA ACG ATA</u> AAA AGC CGA AGC CTT AAA C	5' - AGA ATG TCA AAA ACA ACA TCA <u>ATG CGA TAA TTT TTT TCA TCA CAG</u> CTT TAG AGC GTT TTG CGA T



# The *Escherichia coli* chromosome is organized with the left and right chromosome arms in separate cell halves

Henrik J. Nielsen,<sup>1</sup> Jesper R. Ottesen,<sup>1†</sup>  
Brenda Youngren,<sup>2</sup> Stuart J. Austin<sup>2</sup> and  
Flemming G. Hansen<sup>1\*</sup>

<sup>1</sup>BioCentrum-DTU, Technical University of Denmark, DK 2800 Kgs. Lyngby, Denmark.

<sup>2</sup>Gene Regulation and Chromosome Biology Laboratory, National Cancer Institute, CCR, NCI-Frederick, Frederick, MD 21702-1201, USA.

## Summary

**We have developed a system for the simultaneous labelling of two specific chromosomal sites using two different fluorescent ParB/*parS* systems. Using this, we demonstrate that the two chromosome arms are spatially arranged in newborn cells such that markers on the left arm of the chromosome lie in one half of the cell and markers on the right arm of the chromosome lie in the opposite half. This is achieved by reorganizing the chromosome arms of the two nucleoids in pre-division cells relative to the cell quarters. The spatial reorganization of the chromosome arms ensures that the two replication forks remain in opposite halves of the cell during replication. The relative orientation of the two reorganized nucleoids in pre-division cells is not random. Approximately 80% of dividing cells have their nucleoids oriented in a tandem configuration.**

## Introduction

Although some reports suggest otherwise (Sunako *et al.*, 2001; Bates and Kleckner, 2005), it has long been proposed that the *Escherichia coli* chromosome is segregated progressively as it is replicated (Dingman, 1974). Extensive analysis of the positions of fluorescently labelled markers around the chromosome clearly shows that this principle applies, at least in slow growing MG1655 cells (Nielsen *et al.*, 2006). Under these conditions, newborn cells have the origin of replication at or near the cell centre.

Accepted 17 July, 2006. \*For correspondence. E-mail fgh@biocentrum.dtu.dk; Tel. (+45) 45252505; Fax (+45) 45932809. †Present address: Department of Veterinary Pathobiology, Royal Veterinary and Agricultural University, Stigbøjlen 4, DK-1870 Frederiksberg C, Denmark.

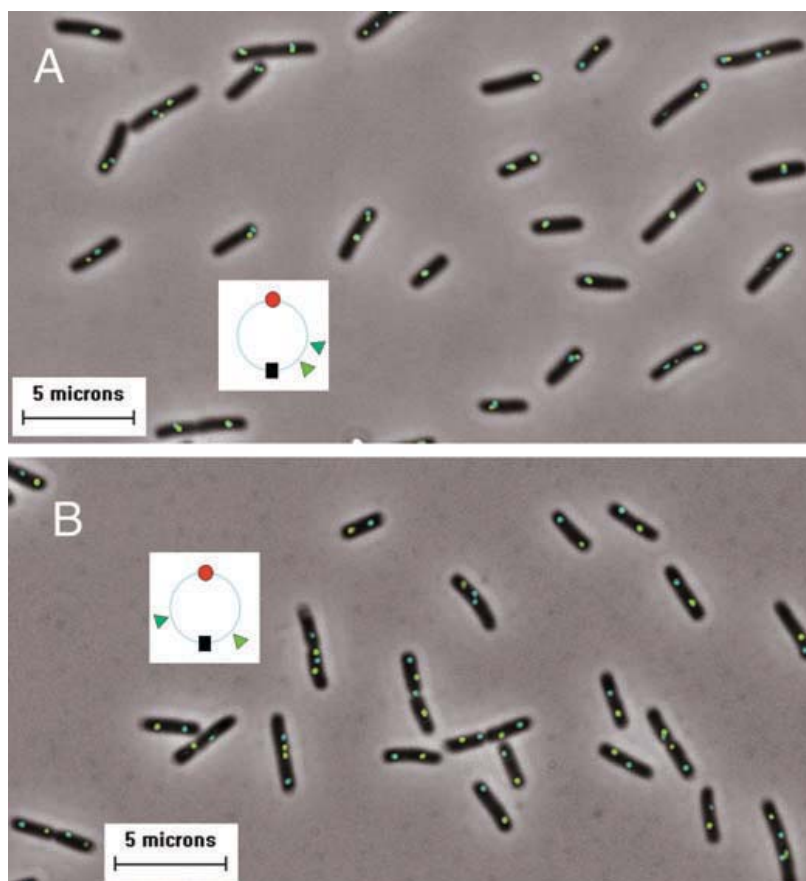
Replication is initiated there. However, as the replication forks progress away from the origin, they dissociate from the cell centre and travel outward along the path of the unreplicated DNA (Yamazoe *et al.*, 2005; Nielsen *et al.*, 2006). The replicated sister duplexes remain close to each other for a while after replication (sister loci cohesion, Nielsen *et al.*, 2006) but are then segregated such that one copy remains approximately in place and the other moves to the opposite half of the cell. The origins are located near the cell quarters in pre-division cells, in readiness for the next round of replication in the following cell cycle. Like the origin of replication, the terminus of the chromosome is located at the cell centre prior to its replication. The duplicated termini remain at the centre until late in the cell cycle. Thus, cell division generally results in newborn cells with the terminus near to the newest cell pole (Niki and Hiraga, 1998; Nielsen *et al.*, 2006).

In our previous study, the bulk of the chromosome appeared to be rather haphazardly organized, with markers occupying a wide variety of possible positions in the nucleoid, both before and after replication and segregation (Nielsen *et al.*, 2006). However, this was shown using a single marked sequence in each strain examined. As it is not possible to tell one end of the cell from the other in the rod-shaped *E. coli* cells, asymmetrically distributed markers could not be assigned to a specific half of the cell. Here, we describe the development of a new method for differentially labelling two specific chromosome sites simultaneously in living cells. The results show that one arm (replichore) of the chromosome is present in one half of the pre-replicative cell and the other in the other half. This implies that, although DNA replication and segregation results in considerable disorder in the nascent nucleoids, some unknown mechanism rearranges the chromosome arms in dividing cells with respect to the cell quarters, so that newborn cells have an orderly separation of the arms. We discuss the probable advantages to the cell gained from this rearrangement.

## Results

### *Detection of two loci simultaneously using two plasmid parS sites of different specificity*

We have previously described a fluorescent labelling system based on the P1 plasmid partition site P1*parS* and



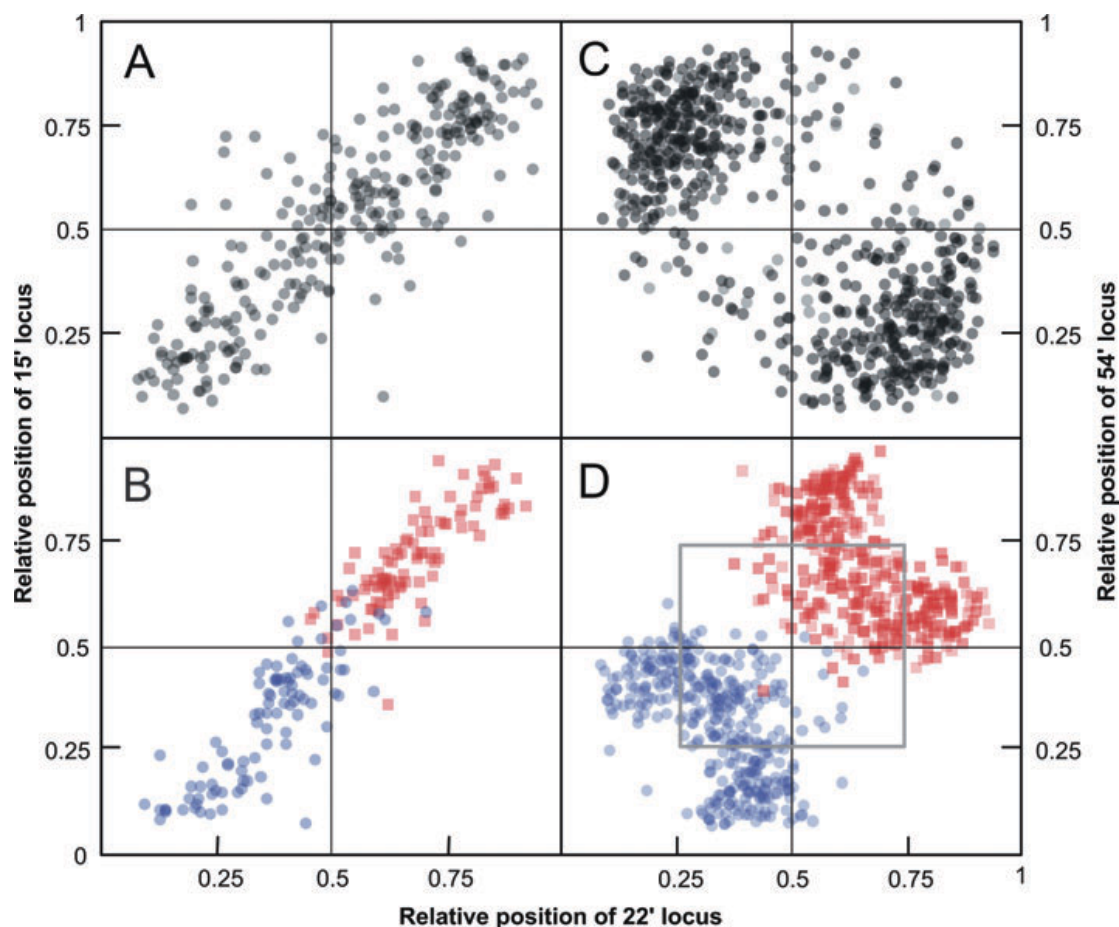
**Fig. 1.** Images of strains FH2973 and FH3518. Fluorescent images captured with a green filter were pseudo-coloured green and fluorescent images captured using a cyan filter were pseudo-coloured cyan and both subsequently overlaid on the phase-contrast image. A shows cells of the FH3518 strain (P1*parS* at 15' and pMT1*parS* at 22') and B shows cells of the FH2973 strain (P1*parS* at 54' and pMT1*parS* at 22'). The insert mini-maps show the chromosomal position of the P1*parS* locus (cyan) and the pMT1*parS* locus (green) in the two strains. The origin and terminus are shown in red and black respectively.

its cognate fluorescent binding protein GFP- $\Delta$ 30ParB (Li *et al.*, 2002; Nielsen *et al.*, 2006). This is derived from the partition system of the P1 plasmid. The P1 partition system is one of a closely related family of such elements found in the plasmids of a variety of bacterial species (Dabrazhynetskaya *et al.*, 2005). We have developed a similar labelling system based on the P1-like partition system of the pMT1 virulence plasmid of *Yersinia pestis*. Strain FH2953 was constructed with a pMT1*parS* site at the 22' position in the chromosome and a P1*parS* site at 54' (chromosomal minutes). This strain was the final recipient in the construction of a plasmid pFHC2973, which produces both CFP-P1 $\Delta$ 30ParB and yGFP-pMT1 $\Delta$ 23ParB proteins (*Experimental procedures*). As the ParB proteins of P1 and pMT1 only recognize the *parS* sites of their own species (Youngren *et al.*, 2000), the cells of the strain contain two types of fluorescent foci: cyan foci marking the position of the DNA containing the P1*parS* site and green foci marking the position of the DNA containing the pMT1*parS* site (Fig. 1B). It should be noted that the fluorescent ParB proteins do not cross-react (form fluorescent foci) in strains carrying *parS* sequences of the opposite specificity (data not shown). The cells were grown in minimal glycerol medium at 32°C.

Under these conditions, initiation occurs at cell birth, and the 22' and 54' regions are duplicated and segregated approximately half way through the cell cycle (Nielsen *et al.*, 2006). Note that most of the smaller cells contain one cyan and one green focus whereas most of the larger cells contain two cyan and two green foci. Thus, the number of foci produced appears to correlate well with the number of marked loci present in the cells. We have previously shown that foci produced by the GFP-P1 $\Delta$ 30ParB/P1*parS* labelling technique are accurate indicators of the number of separate P1*parS* sequences present in each cell (Nielsen *et al.*, 2006). The average number of cyan and green foci, and the distributions of these foci in the population of FH2973 cells, correspond almost exactly to those obtained using a single GFP-P1 $\Delta$ 30ParB/P1*parS* label at each of these two chromosomal sites (data not shown).

#### *Two markers on the same chromosome arm colocalize in the same cell half*

Strain FH3518 has the P1*parS* site inserted at 15' and the pMT1*parS* site inserted at 22' on the chromosomal map (Fig. 1A). These two loci are on the same arm (replichore)



**Fig. 2.** Relative position of foci. The relative distance from the cell pole of cyan and green foci was measured for strain FH3518 (*P1parS* at 15' and *pMT1parS* at 22'; A and B) and strain FH 2973 (*P1parS* at 54' and *pMT1parS* at 22'; C and D, see drawings above A and C). The relative position of the cyan focus is plotted against the relative position of the green focus for each cell. Cells with one pair of foci are shown in A and C, cells with two pairs of foci are shown in B and D. In B and D the blue circles represents the first pair of foci in the cell and the red squares the second. Cells are measured from a random end. Dots inside the grey square in D correspond to foci pairs that have not been rearranged.

of the chromosome and are therefore replicated in the same direction. Using the double label system, we visualized these loci in cells growing in glycerol minimal media at 32°C. The results are analysed in Fig. 2A and B. We see that foci corresponding to both of these loci are regularly found outside the cell centre (outside the central cross in the figure). This is in agreement with previous findings (Nielsen *et al.*, 2006). But we also see that there is a high degree of correlation between the position of the green and cyan foci. In cells with one cyan and one green focus (1c1g), the distance between the two foci varies considerably, but they are generally quite close to each other or are coincident. Moreover, both foci are within the same cell half in the majority (93%) of the cells (the upper right and lower left quadrants in Fig. 2A, see also Fig. 4). Hence the two loci are almost always in the same half of the cell prior to duplication and segregation. After dupli-

cation, the cells end up with one cyan and one green focus in each cell half and, again, these pairs of foci tend to be close together or coincident (Fig. 2B). As shown previously for single markers (Nielsen *et al.*, 2006), the marker pairs in individual cells tend to be asymmetrically distributed, with the pair in one cell half near to the cell centre and the pair in the other half nearer to the outside pole (see Fig. 1A for examples).

#### *Markers on opposite chromosome arms are in opposite cell halves*

As described above, strain FH2973 has its *P1parS* marker at 54' and *pMT1parS* at 22' on the chromosomal map (Fig. 1B). These two loci are on opposite arms (replichores) of the chromosome and are therefore replicated in opposite directions. This strain was analysed in the

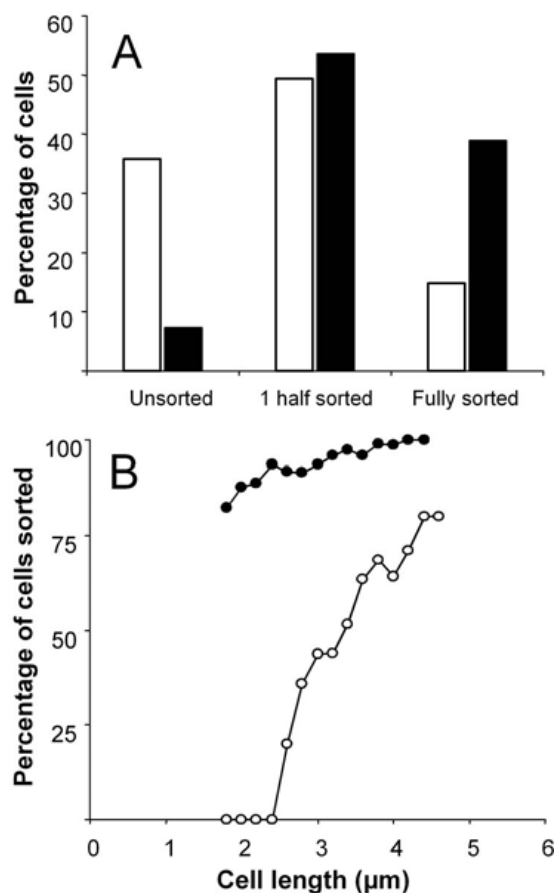
same way as FH3518 and the results are shown in Fig. 2C and D. Here, we see a radically different pattern. In cells with one cyan and one green focus (1c1g cells), the loci hardly ever colocalize (Fig. 2C). Moreover, in a large majority of the 1c1g cells (90%) the cyan and green foci are on opposite sides of the cell centre (the upper left and lower right quadrant in the figure, see also Fig. 4). The few cells with both foci on the same side always have at least one of them close to the cell centre. We conclude that, prior to replication, the two markers that are on the same chromosome arm are in the same cell half, whereas two markers on opposite arms of the chromosome are in opposite cell halves.

*Duplicated loci from opposite arms colocalize temporarily on the inside of the cell quarters*

Figure 2D shows the measurements on cells with two cyan and two green foci (2c2g cells) of FH2973; the strain marked at 22' and 54' on opposite chromosome arms. Note that these cells generally have a cyan and green pair of foci in each cell half, and that cyan and green foci are placed such that they are on opposite sides of the cell quarter positions (dots outside the grey square, Fig. 2D). When these cells divide, they will produce two daughters with a green and cyan focus in each cell half, as is observed for the 1c1g cells.

However, there is also a portion of the 2c2g population that does not show this pattern. These cells have both loci on the inside of the quarter position in one or both cell halves (dot inside the grey square, Fig. 2D). If these cells divided with this configuration they would produce newborn cells with both loci in the same cell half. This we virtually never see (Fig. 2C). Hence, the 2c2g subpopulation appears to represent a stage in which the chromosome is disorganized, and will later be reorganized so that the chromosome arm will be in opposite cell halves in the next generation. To investigate this, we compared a subset of the smaller (youngest) cells in the 2c2g population with a subset of the septated ones that were about to divide (Fig. 3A). The population of smaller 2c2g cells contained a large fraction of cells that have both foci on the inside of the cell quarters. However, the septated cells that are nearer to cell division had far fewer cells of this type. This was observed in several experiments, and was also seen when the smaller 2c2g cells were compared with large ones, irrespective of the state of division. Thus, colocalization of the 22' and 54' loci within the cell quarter positions appears to be an intermediate step. Eventually, the nucleoid will reorganize so that the loci will span the cell quarters and will be on opposite sides of the cell centre after cell division.

As some of the 1c1g cells did have the foci on the same side of the cell centre, we speculated that, occasionally,



**Fig. 3.** Progressive rearrangement of loci.

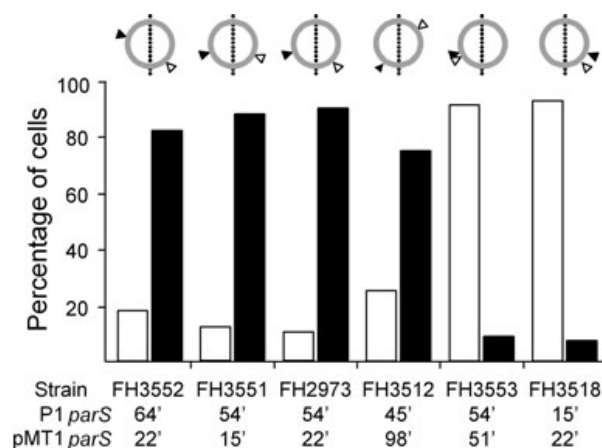
A. The fraction of 'unsorted' FH2973 cells (both the two 22' and the two 54' foci inside the cell quarters), the fraction of cells with one half 'sorted' (one 22' and one 54' focus on each side of the quarter in one half and the 22' and 54' foci inside the quarter in the other half) and the fully 'sorted' cells (one 22' and one 54' focus on each side of the quarter in both cell halves) is shown. This was determined for 83 dividing cells (166 cell halves; black bars) and 83 of the smallest 2c2g non-dividing cells (white bars). B. The fraction of 'sorted' 1c1g cells with the 22' focus and 54' focus in separate cell halves is shown for different cell size classes (filled circles). The fraction of 'sorted' 2c2g half-cells (having the 22' and 54' focus on opposite sides of the cell quarter) is shown for different cell size classes (open circles).

the arranging of the chromosome arms is not completed until after cell division. If that is the case we would expect the proportion of 1c1g cells with both loci on the same side of the cell centre to be maximal in the newborn cells and that the fraction of such cells would decrease as the cells grow. This trend was observed (Fig. 3B).

*The ordering of the chromosome arms extends to other markers*

Figure 4 shows the result of the analysis of four new strains that have the P1parS and pMT1parS sites at dif-





**Fig. 4.** Verification of the chromosome arm arrangement using multiple markers. The location of fluorescent foci in six strains with P1*parS* and pMT1*parS* sites at various chromosomal locations and expressing the cognate *parB* fusion proteins from plasmid pFHC2973 was determined. The percentage of 1c1g cells with one cyan and one green focus located on opposite sides of the cell centre on each is shown by the solid bars. The percentage of 1c1g cells with one cyan and one green focus on the same side of the centre is shown as open bars. The chromosomal positions of the two *parS* sites for the various strains are written below and shown schematically on mini-maps above the histograms. On the mini-maps, the origin-terminus axis is shown as a dotted line with the origin at the top; the positions of the P1- and pMT1*parS* sites are shown as black and white triangles respectively.

ferent positions on the chromosome compared with the two strains described above. The four new strains were constructed as described in *Supplementary material* and carried plasmid pFHC2973 expressing the two respective ParB fusion proteins. When the two *parS*-sequences of different specificity were placed on the same chromosome arm (strains FH3518 and FH3553), the cyan and green foci were found on the same side of the cell in the majority of the 1c1g cells. When the two *parS*-sequences were on opposite arms of the chromosome, the cyan and green foci are found in opposite cell halves (strains FH2973, FH3512, FH3551 and FH3552). In sum, the observed markers cover the region from 45 min to 64 min (46% of the left chromosome arm) and from 89 min to 21 min. (39% of the right arm). We conclude that the spatial organization of the chromosome arms into opposite halves of the cell involves about half of the chromosome, and presumably more.

#### *Duplicated chromosome arms are usually oriented in a tandem repeat configuration*

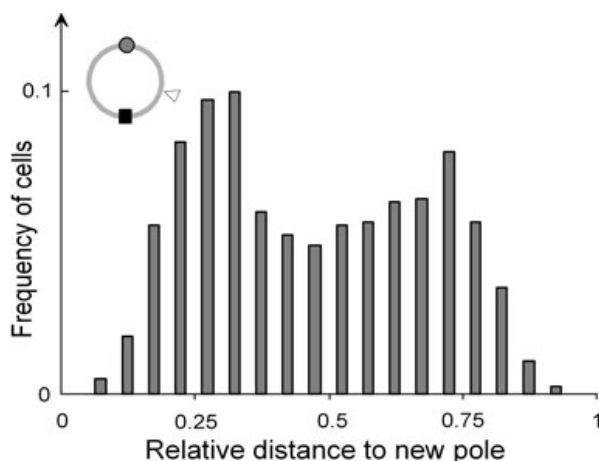
It was previously reported that intermediate loci (i.e. loci that are separated from the origin and terminus of replication) tend to be asymmetrically positioned in the cell (i.e. one near the pole and one near the centre, Wang

*et al.*, 2005; Nielsen *et al.*, 2006). The discovery that the chromosomal arms are in separate cell halves can provide an explanation for this observation. The duplication of the chromosome and the arranging of the chromosome arms could lead to two different relative orientations of the nucleoids in dividing cells. They could be symmetrically arranged, in which case the distribution of intermediate markers would be symmetrical about the cell centre. Alternatively, they could be arranged in tandem, in which case intermediate marker distributions would show a distinct asymmetry with markers tending to have one copy close to the pole and its sister locus just on the other side of the middle. With double labelling, these cases can be distinguished. We looked specifically at the dividing cells in the FH2973 population for the order of the foci. On examining 65 fully sorted, dividing cells (of 226 total 2c2g cells), 54 (83%) had the foci in the cyan, green, cyan, green configuration. The remainder was equally divided between cyan, green, green, cyan and green, cyan, cyan, green configurations. These distributions were reproducible between several independent experiments. A similar result was obtained with strain FH3551 (P1*parS* at 54' and pMT1*parS* at 15') where 74% of fully sorted 2c2g cells were in the parallel cyan, green, cyan, green configuration. We conclude that in most, but not all of the cells, the arranging of the chromosome arms results in a parallel configuration of the two nucleoids prior to cell division.

#### *Sister cells are not usually identical: the majority has opposite nucleoid organizations*

Although the new pole of the cell cannot be distinguished visually, its identity can be inferred from the position of the terminus. The 33' marker should lie close to the new pole in newborn cells as the terminus is held near the cell centre as the cell divides (Niki and Hiraga, 1998; Lau *et al.*, 2003; Bates and Kleckner, 2005; Nielsen *et al.*, 2006). After a while, the terminus migrates to the cell centre. However, in all cells that have an asymmetrically placed 33' marker, the marker should be adjacent to the new pole (Lau *et al.*, 2003; Nielsen *et al.*, 2006).

If the majority of dividing cells have the two nucleoids in tandem configuration, division should produce two daughters, one of which has the left arm of the chromosome near the new pole and the other having the right arm near the new pole. To confirm this, we looked at the strain FH3550 that has the P1*parS* at 33', close to the terminus and the pMT1*parS* at 14' on the chromosomal map (Fig. 5). The position of the 14' locus compared with the 33' (new pole-proximal) marker is shown in Fig. 5. As can be seen, there is no correlation between the position of



**Fig. 5.** Location of the 14 min marker with respect to the new pole. The 1c1g cells of strain FH3550 with P1*parS* at 33' and pMT1*parS* at 14' were oriented with respect to the terminus focus, which is adjacent to the new pole of the cell. The relative distance from this new pole to the 14' focus was then measured and the distribution for all cells plotted as a histogram (1003 cells measured). It is evident that it is equally probable to find the 14' locus close to either the new or the old pole. The insert mini-map shows the chromosomal position of the 14' marker (white) and the terminus marker (black); the origin is shown in grey.

the chromosome arm carrying the 14' *parS*-sequence and the new pole in this plot. This is in agreement with a tandem configuration of the chromosomes in most dividing cells, as the arm carrying the 14' marker would be adjacent to the new pole in one cell of a newborn pair, and adjacent to the old pole in the other.

It has generally been assumed that both daughter cells produced by binary fission in bacteria are more or less identical. In the context of our present finding, this would imply that the two nucleoids are oriented symmetrically and that newborn sisters both have a particular arm of the chromosome adjacent to the new pole. This is clearly not the case in most cells. The two daughters are usually radically different in this respect.

## Discussion

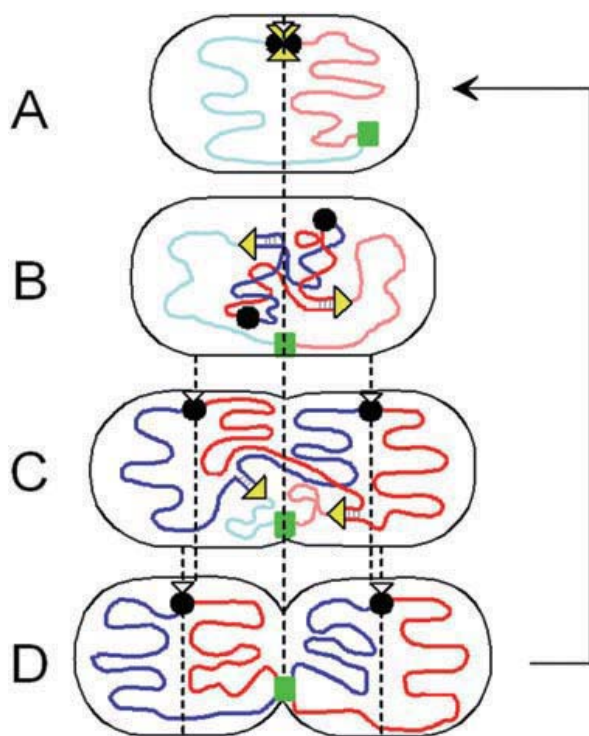
The *E. coli* chromosome replicates bidirectionally, with each half of the chromosome replicated by a different replication fork. How are these two replicating regions arranged in the cell? The possibility that the forks remain together throughout replication as a central factory now appears to be eliminated. The forks follow separate paths as they progress in opposite directions away from the origin (Bates and Kleckner, 2005; Yamazoe *et al.*, 2005; Nielsen *et al.*, 2006).

One could imagine two extreme cases. In one, the forks follow the path of a randomly arranged chromosome within the nucleoid. Thus replication would take place

throughout the nucleoid with the forks frequently passing each other as replication takes place. In the other extreme, the two arms of the chromosome (replichores) would be arranged in opposite halves of the cell and each replication fork would be constrained to its own cell half. This arrangement would greatly simplify the topological problems encountered while replicating and segregating the chromosome. As the replication forks follow the path of the DNA, they would remain in opposite cell halves and not cross-over the centre line or pass across the path of the opposing fork. Thus the potential for knotting and entanglement of the replication products would be much reduced.

In this study, we show definitive evidence that the left and right chromosome arms are indeed organized on opposite sides of the cell centre. This is in agreement with the recent observation that the terminus region of the chromosome is organized such that markers to the left of the terminus generally lie in the opposite cell half to those to the immediate right of the terminus (Wang *et al.*, 2005). The fact that the chromosome of slow growing cells is organized with one arm in each cell half has been previously hinted by the fact that the replication forks appear to migrate to opposite cell halves in slow growing cells during replication and seldom localize in the same half once they are separated (Brendler *et al.*, 2000; Bates and Kleckner, 2005; Yamazoe *et al.*, 2005).

We show here that the left arm/right arm organizational plan extends to much of the chromosome, and that this is achieved by a previously unsuspected DNA rearrangement mechanism. It appears to take relatively disorganized products of nucleoid replication and segregation and arranges the nucleoid arms with respect to the cell quarter positions. After duplication, the loci of the opposite arms often localize together in the space between the quarter and the centre of the cell. The chromosome arms are then actively sorted out with respect to the cell quarters (Fig. 6). This reorganization usually takes place before cell division, but some cells divide before the rearrangement is completed. We never find pre-division cells with both loci from opposite arms together on the outside of the quarter. Hence the two arms are initially put on the same side of the quarter (the inside) and the rearrangement mechanism subsequently puts one and only one arm on the outside. We find that the orientation of the arm arrangement in one cell half is dependent on the orientation of the other. Usually, but not always, the two arranged chromosomes are arranged in a tandem configuration so that the left arm of the chromosome is on the outside of the quarter in one cell half and on the inside in the other half; and vice versa for the right arm (Fig. 6). This discovery explains the high number of cells with asymmetric distributions of intermediately replicated loci in single colour studies



**Fig. 6.** Chromosome rearrangement model.

A. At initiation, the origin region of the chromosome (black circles) is at the cell centre, and the replication forks (yellow triangles) form there. The two arms (light blue and pink lines) of the chromosome are arranged either side of the cell centre. The terminus (green square) is near the new cell pole.

B. At mid-replication, the forks have dissociated from the cell centre and are following the path of the DNA template. The newly replicated DNA products (dark blue and red) stay together for a while (sister loci cohesion, grey hatching) and then segregate to opposite sides of the cell centre. This DNA is relatively disorganized and resides in the central half of the cell.

C. As replication progresses, the new origins attach to the cell quarter positions and the two arms of each nascent chromosome are sorted out so that the arms lie on either side of the cell quarters.

D. At termination, the origins are at the quarter positions, and the terminus and forks are at the cell centre. The arms (replichores) of the two new chromosomes are arranged with respect to the cell quarters. After the following post-replicative period (D-period), cell division will restore the starting state of the cycle.

(Nielsen *et al.*, 2006). The rearrangement mechanism itself may bias the relative nucleoid configurations to the tandem arrangement. Alternatively, the rearrangement mechanism might be indifferent to which arm is to be moved, but the DNA segregation mechanism biases the placement of newly replicated DNA in the central cell quarters in such a way that a tandem configuration becomes likely.

In any case, it is clear that a previously unsuspected mechanism exists for DNA arrangement at the cell quarters. The nature of this mechanism, the information present on the chromosome arms to direct it, and the

proteins present at the quarters that we presume to carry it out, remain to be identified.

## Experimental procedures

### Bacterial strains and plasmids

The strains used in this study are all derivatives of strain FH2926, a  $\Delta lacI-lacA$  derivative of MG1655, carrying the different P1*parS* sequence and the pMT1*parS* sequence at various chromosome positions. The strains were constructed as described in *Supplementary material*. The construction of plasmid pFHC2973, which carries both the P1-*parB* gene and the pMT1-*parB* gene fused to the genes for the fluorescent proteins CFP and yGFP, respectively, is also described in *Supplementary material*.

### Growth conditions, microscopy and measurements

The general methods employed were as previously described (Nielsen *et al.*, 2006).

## References

- Bates, D., and Kleckner, N. (2005) Chromosome and replisome dynamics in *E. coli*: loss of sister cohesion triggers global chromosome movement and mediates chromosome segregation. *Cell* **121**: 899–911.
- Brendler, T., Sawitzke, J., Sergueev, K., and Austin, S. (2000) A case for sliding SeqA tracts at anchored replication forks during *Escherichia coli* chromosome replication and segregation. *EMBO J* **19**: 6249–6258.
- Dabrazhynetskaya, A., Sergueev, K., and Austin, S. (2005) Species and incompatibility determination within the P1*par* family of plasmid partition elements. *J Bacteriol* **187**: 5977–5983.
- Dingman, C.W. (1974) Bidirectional chromosome replication: some topological considerations. *J Theor Biol* **43**: 187–195.
- Lau, I.F., Filipe, S.R., Soballe, B., Okstad, O.A., Barre, F.X., and Sherratt, D.J. (2003) Spatial and temporal organization of replicating *Escherichia coli* chromosomes. *Mol Microbiol* **49**: 731–743.
- Li, Y., Sergueev, K., and Austin, S. (2002) The segregation of the *Escherichia coli* origin and terminus of replication. *Mol Microbiol* **46**: 985–996.
- Nielsen, H.J., Li, Y., Youngren, B., Hansen, F.G., and Austin, S. (2006) Progressive segregation of the *Escherichia coli* chromosome. *Mol Microbiol* **61**: 383–393.
- Niki, H., and Hiraga, S. (1998) Polar localization of the replication origin and terminus in *Escherichia coli* nucleoids during chromosome partitioning. *Genes Dev* **12**: 1036–1045.
- Sunako, Y., Onogi, T., and Hiraga, S. (2001) Sister chromosome cohesion of *Escherichia coli*. *Mol Microbiol* **42**: 1233–1241.
- Wang, X., Possoz, C., and Sherratt, D.J. (2005) Dancing around the divisome: asymmetric chromosome segregation in *Escherichia coli*. *Genes Dev* **19**: 2367–2377.
- Yamazoe, M., Adachi, S., Kanaya, S., Ohsumi, K., and Hiraga, S. (2005) Sequential binding of SeqA protein to

nascent DNA segments at replication forks in synchronized cultures of *Escherichia coli*. *Mol Microbiol* **55**: 289–298.

Youngren, B., Radnedge, L., Hu, P., Garcia, E., and Austin, S. (2000) A plasmid partition system of the P1-P7par family from the pMT1 virulence plasmid of *Yersinia pestis*. *J Bacteriol* **182**: 3924–3928.

### Supplementary material

The following supplementary material is available for this article online:

**Appendix S1.** Strain construction and construction of plasmids carrying two different ParB fluorescent proteins of different colours.

**Figure S1.** Structure of plasmids for generating PCR products for recombineering.

**Figure S2.** Construction of plasmids carrying genes for fluorescent ParB proteins of different specificities.

**Table S1.** Oligonucleotide sequences.

This material is available as part of the online article from <http://www.blackwell-synergy.com>.



## Supplementary material

### Strain construction

Strain FH2926 has the *lac* operon deleted, and was constructed as follows. First a PCR fragment carrying the *cat* gene bracketed by two *loxP* sites was synthesized, using the oligonucleotide pair 15.08-15.09 (Table S1) with plasmid pHJN2 (Figure S1) as template. This PCR fragment was used as template for a new PCR reaction using the oligonucleotide pair 22.02-22.03 (Table S1). The PCR fragment was transformed into an MG1655 strain carrying plasmid pKD46 (Datsenko and Wanner, 2000) by electroporation, selecting for chloramphenicol resistance and screening for loss of a functional *lac* gene. PCR was used to check the size of the *cat* insert by using oligonucleotide primers flanking the *lac* operon. The *cat* gene was removed by P1 Cre-mediated recombination between the two *loxP* sites by using plasmid pFHC2938. This plasmid is a derivative of pKD46. First the *bla* gene was inactivated by inserting a *AatII-PstI* restriction fragment carrying the *tet* gene of pBR322 into pKD46 restricted with *AcI* (plasmid pFHC2819). Second, a PCR generated *cre* gene (oligonucleotide pair 13.07-13.08, Table S1) was isolated via an intermediate vector and was recovered as a *SacI-BspEI* fragment. This was used to replace the *SacI-XmaI* fragment of pFHC2819 (and of pKD46) which

carried the  $\lambda$  *gam*, *bet*, and *exo* genes, resulting in plasmids pFHC2839 and pFHC3220, respectively. The plasmid construction was carried out in the progenitor of FH2926, which had lost the *cat* gene in the process, and was then cured for pFHC2938.

Strain FH2926 carrying pKD46 (=FH2927) was used as recipient for recombineering the pMT1 *parS* site into several different positions on the chromosome. A plasmid with the pMT1 *parS* site was constructed as follows. First an oligonucleotide linker consisting of the two oligonucleotides 15.10 and 15.11 (Table S1) was ligated into the *BglII-NdeI* sites of pHJN2. Second a PCR fragment carrying the pMT1 *parS* sequence was synthesized with the primers 18.12 and 18.13 (Table S1) and plasmid pALA1840 (Youngren *et al.*, 2000) as template. After restriction with *BamHI* and *BlnI*, the fragment was inserted in the modified pHJN2 plasmid that had been cut with the same enzymes, resulting in plasmid pFHC3228 (Figure S1). The PCR fragment used was synthesized in two steps as described above. First the oligonucleotide pair 15.08-15.09 was used to synthesize a PCR fragment which could be used as template for synthesizing the various PCR fragments using oligonucleotides which would direct the PCR fragment to be recombineered into the chromosome at the desired positions. In this way a number of strains with the pMT1 *parS* sequence inserted at different positions were constructed. A number of strains which, in addition to the pMT1 *parS* sequence, carried the P1

*parS* sequence (Table S2) were constructed by P1 transduction using lysates of strains described earlier (Nielsen *et al.*, 2006). In most cases the *cat* gene was removed by Cre-mediated recombination between *loxP* sites.

It should be noted that the two plasmids pKD46 ( $\lambda$  *gam*, *bet*, and *exo*) and pFHC2938 (P1 *cre*) carry the same pSC101 temperature sensitive replicon and thus are incompatible. Therefore, transforming pFHC2938 into a strain carrying pKD46 selecting for tetracycline resistance leads to loss of pKD46 and loss of the *cat* gene. Retransformation of such a strain with pKD46 leads to loss of pFHC2938 and

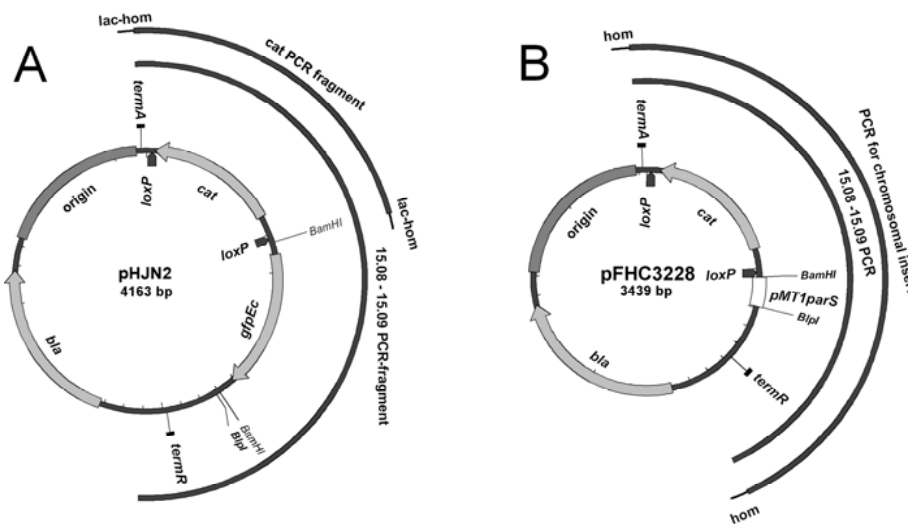


Figure S1. Structure of plasmids for generating PCR-products for recombineering. The plasmids were used to generate the PCR fragments shown by the arcs outside the plasmids. The inner arc was used as the template to generate DNA which could be used for recombineering due to 40-50 bp homology (hom and lac-hom ends) to the *E. coli* chromosome. Plasmid pFHC3228 is a derivative of pHJN2 carrying the pMT1 *parS* sequence. *termA* and *termR* are the positions of an artificial and an *rpoC* terminator sequence, respectively. Only restriction enzyme sites used in the construction of pFHC3228 from pHJN2 are shown.

pFHC2973 was derived from pALA2705 (Li *et al.*, 2002). It has two modified *parB* genes transcribed from the same promoter. A P1*parB* gene, fused to an *E. coli* codon-optimized *cfp* gene is followed by a pMT1 *parB* gene fused to a novel *E. coli* codon-optimized variant of a *gfp* gene where the protein (which we call yGFP to distinguish it from YFP) does not emit light at the wavelength normally used to record the fluorescence of CFP (480 nm). This plasmid and similar derivatives was constructed as follows. Plas-mid pFHC2191 (GenBank Acc. No. AF325903) was restricted with *Bst*API and *Aat*II and a linker fragment (oligonucleo-tides 22.06 and 22.07, Table S1) was inserted between the sites to provide an *Eco*RI site necessary for fusing the codon optimized *gfp* gene to the P1 *parB* gene. The *gfp* gene from the resulting plasmid was excised as a *Aat*II-*Nde*I fragment and was inserted into pFHC2102 (Atlung and

Vector pACYC184-derived plasmids, compatible with the pBR322 replicon and carrying the genes for the fluorescent proteins fused to the pMT1 *parB* gene with a deletion of the codons for 23 N-terminal amino acids were also constructed. Plasmid pFHC3224, which carries an arabinose-controlled codon-optimized *gfp* gene fused to the gene for the pMT1- $\Delta$ 23-ParB protein

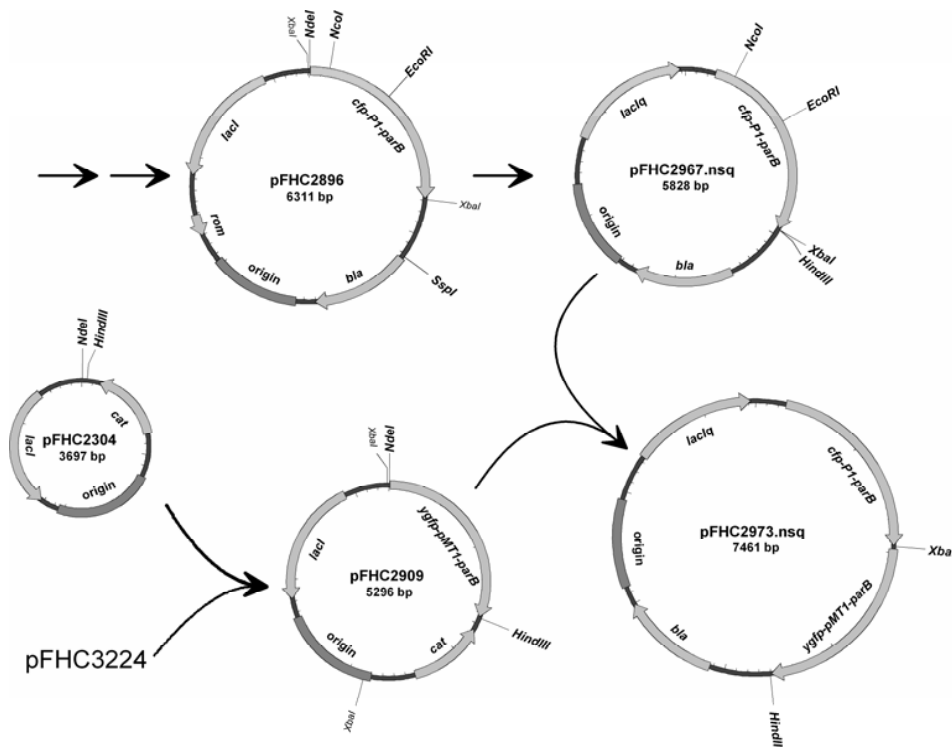


Figure S2. Construction of plasmids carrying genes for fluorescent ParB proteins of different specificities. Only relevant restriction enzyme sites are shown. See text for details.

was restricted with *NdeI* and *HindIII* and inserted in the pACYC184 derived plasmid pFHC2304 (Figure S2) to produce plasmid pFHC2907. Plasmids pFHC2908 (CFP, Figure S2) and pFHC2909 (yGFP) were constructed by exchanging the chromophore region. Finally, plasmids carrying two genes for the different fluorescent proteins fused to different ParB proteins were constructed using restriction enzymes *HindIII* and *XbaI*. Plasmid pFHC2973 (Figure S2) carries the genes for CFP-P1-ParB and yGFP-pMT1-ParB. Plasmid pFHC2972 carries the genes for CFP-P1-ParB and GFP-pMT1-ParB. And plasmid pFHC2974 carries the genes for yGFP-P1-ParB and CFP-pMT1-ParB.

## References

- Atlung, T., Hansen, F.G. (2002) Effect of different concentrations of H-NS protein on chromosome replication and the cell cycle in *Escherichia coli*. *J Bacteriol* **184**: 1843-1850.
- Datsenko, K.A., Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**: 6640-6645.
- Li, Y., Sergueev, K., and Austin, S. (2002) The segregation of the *Escherichia coli* origin and terminus of replication. *Mol Microbiol* **46**: 985-996.
- Nielsen, H.J., Li, Y., Youngren, B., Hansen, F.G., and Austin, S. (2006) Progressive segregation of the *Escherichia coli* chromosome. *Mol Microbiol* **In press**: doi:10.1111/j.1365-2958.2006.05245.x.
- Youngren, B., Radnedge, L., Hu, P., Garcia, E., and Austin, S. (2000) A plasmid partition system of the P1-P7par family from the pMT1 virulence plasmid of *Yersinia pestis*. *J Bacteriol* **182**: 3924-3928.

**Table S1.** Oligonucleotide sequences<sup>1</sup>

Name	Primer 1	Primer 2	
15.08-15.09	AGGAAGCGGAATTCCGGACC	CTCGAGATGCAGAAGACGCA	Used to make a primary template PCR-fragment from pHJN2 and similar plasmids
22.02-22.03	gtagatcgctgaacttgtaggcctgataa gcgagcgtatcaggcaattAGGAAGCGG AATTCCGGACC <sup>3</sup>	cgcaaaacctttcgcggtatggcatgat agcgcccggaagagagatcaattGAAGCTT ACGCGTGGTACCG <sup>3</sup>	Oligomers to delete the lac operon using PCR fragment of made with 15.08-15.09 on pHJN2 as template yielding strain FH2926.
13.07-13.08	CAGGTAAAGGct (1) AGCCGATCCTG <sup>2</sup>	ATTATAGCAAT (g) CATTACGCGTTAA TGGC <sup>2</sup>	Used to amplify the cre gene of bacteriophage P1
15.10-15.11	GATCTGCGGCCGCGTGCCTCTAGAGCA	TATGCTCTAGAGTGCACGCGGCCGCA	Adapter to modify pHJN2
22.06-22.07	CGAATTCCTTTATACAGCTCATGCATGCC	ATGCATGAGCTGTATAAAGAATTCGACG T	Adapter for introducing an <i>EcoRI</i> site in pFHC2191
22.26-22.27	cccatttttgcgatggatctccgcggtacc gggataggcttctataccaatAGGAAGCG GAATTCGGGACC <sup>3</sup>	Acattaggccctggttgcaatgttcctg gttatgcccgaaccacgataggCTCGAGA TGCAGAAGACGCA <sup>3</sup>	Oligomers to insert pMT1 parS site at 98.1 min in strain FH2978 (FH3512).
22.28-22.29	accgatcgctataacgagcgacgctgggg tgccggttttggcctgtcgcgAGGAAGCG GAATTCGGGACC <sup>3</sup>	cgccagcggtcgccaggtactttcccat ccgtatccggcaatcggttcccCTCGAGA TGCAGAAGACGCA <sup>3</sup>	Oligomers to insert pMT1 parS site at 14.1 min in strain FH2952. (FH3551).
22.30-23.01	gatgcaggatgtcaccacagacgcatggc caacctggccggtaaaactggAGGAAGCG GAATTCGGGACC <sup>3</sup>	cgataaacagcactgaagtgggtaattgt cacaccatacgctgtttttgcCTCGAGA TGCAGAAGACGCA <sup>3</sup>	Oligomers to insert pMT1 parS site at 22 min in strain FH2973, 2981, 3505. (FH2973, 3552, 3518).
24.10-24.11	caatacataacagaaacctgaaacacaaa acggcagcccttgagctgccgAGGAAGCG GAATTCGGGACC <sup>3</sup>	tgatgagatcgatagcgactaaatcgct tcagtttcacaactgacagaatCTCGAGA TGCAGAAGACGCA <sup>3</sup>	Oligomers to insert pMT1 parS site at 14.1 min in strain FH3506. (FH3553).

<sup>1</sup> All sequences are presented in the 5' to 3' direction.

<sup>2</sup> Small letters represent bases which do not fit the template sequence. A number in parentheses represents a one-base deletion compared to the template sequence; a small letter in parentheses represents a one-base insertion.

<sup>3</sup> Small letters represent bases which do not fit the template sequence but fits the chromosome for facilitating the recombineering process. The capital letters fits the template sequence of plasmid pHJN2 and pHJN2-derived plasmids.